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MicroRNA expression profiles in hereditary breast cancer

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MicroRNA expression profiles in hereditary breast cancer

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HUMAN GENETICS GROUP

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CERTIFICA:

Que Doña Miljana Tanic, Máster en Biología Molecular y Celular por la Universidad Autónoma de Madrid, ha realizado la presente Tesis Doctoral **"MicroRNA expression profiles in hereditary breast tumors"** y que a su juicio reúne plenamente todos los requisitos necesarios para optar al **Grado de Doctor en Biociencias Moleculares**, a cuyos efectos será presentado en la Universidad Autónoma de Madrid. El trabajo ha sido realizado bajo mi dirección, autorizando su presentación ante el Tribunal Calificador.

Y para que así conste se extiende el presente certificado,

Madrid, julio 2012

V^{o} B^{o} del Director de la Tesis:

Dr. Beatriz Martínez-Delgado, Investigadora de grupo de Genética del Cáncer Humano del Centro Nacional de Investigaciones Oncológicas (CNIO)

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Dedicated to my family

Мом деди

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I would like to express my most sincere gratitude to all the people that made part of my life over these four years and contributed to the making of this work.

Yours truly,

Miljana

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RESUMEN

El cáncer de mama sigue siendo en nuestros días la segunda causa de muerte por cáncer en mujeres y el cáncer más común entre las mujeres, siendo por tanto una entidad bajo una intensa investigación. El cáncer de mama hereditario comprende aproximadamente el 5-10% de todos los casos de cáncer de mama. Las mutaciones germinales en los dos genes de alta susceptibilidad más importantes, *BRCA1* y *BRCA2*, junto con las mutaciones en otros genes de susceptibilidad pueden explicar sólo el 40% de los casos. Por lo tanto, la etiología de los casos de cáncer de mama que se presentan en familias de alto riesgo no atribuible a mutaciones en alguno de los genes de susceptibilidad conocidos, tumores de tipo BRCAX, se desconoce. Estos tumores son muy heterogéneos en cuanto a su morfología, histología, perfiles moleculares y comportamiento clínico. En los últimos años han tenido una gran importancia la identificación de los perfiles de expresión de microRNA (miRNA) para la clasificación de los cánceres humanos, y se ha descrito la desregulación de miRNAs implicada en importantes procesos biológicos. En concreto, en el cáncer de mama, los perfiles de expresión de miRNA se asocian a subtipos específicos o características biológicas, como la presencia de receptores hormonales, el estado de HER2, las metástasis, la progresión o la proliferación. Sin embargo, se sabe muy poco sobre el papel de los miRNAs en la patogénesis del cáncer de mama hereditario. Con el propósito de delinear los mecanismos responsables de la tumorigénesis del cáncer de mama hereditario hemos adoptado una variedad de enfoques que incluyen análisis de expresión de miRNAs de forma masiva o individual, análisis de mRNA y expresión de proteínas así como estudios funcionales.

De esta forma hemos establecido el patrón de expresión global de miRNAs y de mRNAs después de la reconstitución del gen *BRCA1* en la línea celular HCC1937, posteriormente hemos integrado estos datos para así modelar parejas de miRNAs y genes regulados por estos diferencialmente expresados definir redes funcionales afectadas en estas células. Nuestros datos sugieren un papel de BRCA1 en la modulación de una serie de miRNAs e indirectamente de cientos de genes, a su vez promueven la represión de diferentes vías de señalización como las mediadas por NF-kB y MAPK. Hemos identificado el gen TRAF2 como nueva diana para los miR-146, miR-99 y miR-205, y hemos demostrado que estos miRNAs son suficientes para modular la actividad de la vía de NF-kB en células de cáncer de mama. Por último, nuestros datos ponen de manifiesto la importancia de un enfoque integrado para el estudio de expresión de miRNAs y genes, para la identificación de vías de señalización alteradas y genes candidatos que pudieran ser importantes en el desarrollo del tumor. La pérdida de expresión específica de ciertos miRNAs encontrada en los tumores primarios sugiere su papel en la tumorigénesis así como podrían ser importantes para el desarrollo de futuras terapias

En la actualidad no existen prácticamente estudios que describan de forma amplia la expresión de miRNAs en los tumores de mama hereditarios. Mediante el uso de microarrays de sondas LNA hemos establecido los perfiles de miRNAs en una serie de carcinomas primarios de mama hereditario de mama así

como de un grupo de los tejidos de mama normales, y de tumores de mama esporádicos. El análisis de estos perfiles ha puesto de manifiesto una serie de miRNAs que se expresan de forma aberrante en los carcinomas de mama hereditarios, incluyendo la disminución de expresión de ciertos miRNAs previamente implicadas en el cáncer de mama esporádico, lo que implica una vía común de la progresión del tumor, independientemente de los sucesos iniciadores. La mayor parte de los miRNAs diferencialmente expresados mostraron en su mayoría una reducción significativa de la expresión en los tumores comparando con el tejido normal. Uno de estos miRNAs encontrados es el miR-30c, que potencialmente contribuye a la formación de tumores malignos de mama mediante la liberación de la supresión del gen KRAS, lo que sugiere que este miRNA, y probablemente de forma conjunta con otros miRNAs, puedan funcionar como supresores tumorales en cáncer de mama a través de la regulación del gen KRAS y por tanto de la vía de señalización de MAPK.

Por otra parte hemos establecido una clasificación de los diferentes subtipos de tumores de mama hereditarios, BRCA1, BRCA2 y BRCAX, así como de tumores de mama esporádicos y tejidos normales en función de sus perfiles de miRNA de expresión revela una clara separación entre los grupos. Hemos identificado un gran número de miRNAs expresados diferencialmente entre los tumores de mama con mutación en BRCA1/2 y los que no tiene mutación, que puede servir como punto de partida para el desarrollo de biomarcadores de diagnóstico que potencialmente pudieran ser útiles para discriminar entre los tumores con mutación en BRCA1/2 y facilitando la identificación de las personas en situación de riesgo de ser portadores de la mutación. Además los perfiles de expresión de los tumores BRCAX revelaron una heterogeneidad clara, con 4 subgrupos definidos por aparentes firmas de miRNAs específicos y diferentes características histológicas que sugieren diferentes vías en la evolución de estos tumores.

En resumen, nuestros resultados abren nuevas perspectivas en los mecanismos que están detrás de la desregulación BRCA1 y aportan nuevas ideas sobre la heterogeneidad molecular de los tumores de mama hereditarios que podrían ser la base para futuras investigaciones en el desarrollo de biomarcadores de diagnóstico y la comprensión de la etiología de los tumores BRCAX.

SUMMARY

Breast cancer continues to be the second leading cause of cancer deaths in women today and being the most common cancer among woman, it is an object of intensive research. Hereditary breast cancer comprises approximately 5-10% of all breast cancer cases. Germline mutations in two high susceptibility genes BRCA1 and BRCA2, along with mutations in other susceptibility genes can account for only up to 40% of cases. Thus, the etiology of breast cancer cases that arise in high-risk families not attributable to mutations in any know breast cancer susceptibility gene, BRCAX-type tumors, is unknown. These tumors are highly heterogeneous in terms of their morphology, histology, molecular profiles and clinical outcomes. In recent years microRNA (miRNA) expression profiling calls great attention for classification of human cancers, and microRNA deregulation has been implicated in important biological processes. Specifically, in breast cancer, miRNA expression profiles were associated to specific subtypes or biological features such as hormone receptor, HER2 status, metastasis, progression or proliferation. However, very little is known regarding the role of microRNAs in hereditary breast cancer pathogenesis. With the purpose of delineating the mechanisms responsible for hereditary breast tumorigenesis we have adopted a variety of approaches including high- and low-throughput microRNA, mRNA and protein expression analysis and functional studies.

We have established the global miRNA and mRNA expression pattern after *BRCA1* reconstitution in HCC1937 cells, and integrated the data to model differentially expressed miRNA and genes into functional networks. Our data suggest a role for BRCA1 in modulating a number of miRNAs and indirectly hundreds of genes, in turn promoting the repression of NF-κB and MAPK signaling pathways. We have identified *TRAF2* as a novel target gene for miR-146, miR-99 and miR-205, and shown that these miRNAs are sufficient to modulate NF-κB pathway activity in breast cancer cells. Finally, our data underscores the importance of an integrated approach to study of miRNA and gene expression, for identification of altered signaling pathways and promising candidate genes. Down-regulation of these miRNAs also in primary tumors suggests their role in tumorigenesis and as potential targets for future treatment development.

At present there are no studies describing miRNA expression in hereditary breast tumors. We have performed LNA-based microRNA microarray profiling in a series of primary hereditary breast carcinomas and normal breast tissues. The microRNA analysis revealed a series of aberrantly expressed microRNAs in hereditary breast carcinomas including downregulation of several miRNAs previously implicated in sporadic breast cancer, implying a common pathway of tumor progression irrespective of the initiating events. These miRNAs mostly showed significant reduced expression in tumors comparing to normal breast tissue. One of these miRNAs, miR-30c, potentially contributes to breast malignancy formation through release of KRAS suppression suggesting that this miRNA, and likely other miRNAs also targeting KRAS/MAPK signaling, may function as tumor suppressors in breast cancer.

Classification of breast tumors from different genetic subtypes, BRCA1, BRCA2 and BRCAX, sporadic breast carcinomas and normal tissues based on their microRNA expression profiles revealed clear segregation between the groups. We have identified a large number of miRNAs differentially expressed between *BRCA1/2* mutation positive and mutation negative breast tumors, which can serve as a starting point for the development of biomarkers of diagnosis that would potentially discriminate between mutation positive tumors and facilitate identification of individuals at risk of being mutation carriers. Hierarchical clustering of BRCAX tumors revealed clear heterogeneity, with 4 apparent subgroups defined by specific miRNA signatures and different histological characteristics suggesting different paths in tumor evolution.

In summary, our findings bring new insights in the mechanisms behind BRCA1 deregulation and new ideas on the molecular heterogeneity of hereditary breast tumors that hold the basis for future research into development of diagnostic biomarkers and understanding the etiology of BRCAX tumor.

ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
BCL2	B-cell lymphoma 2
BRCA1	Breast-cancer susceptibility gene 1
BRCA2	Breast-cancer susceptibility gene , aka. FANCD1, Fanconi anemia group D1 protein
BRCAX	Breast-cancer susceptibility gene X
BRIP1	BRCA1 interacting protein C-terminal helicase 1, aka. BACH1, BRCA1-binding helicase-like protein
BSA	Bovine serum albumin
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CGH	Comparative genomic hybridization
CHEK2	Checkpoint-like protein CHK2
CIP	Calf intestinal phospatase
CK 5	Cytokeratin 5
CK 8	Cytokeratin 8
c-MYC	myc proto-oncogene protein
Ct	Threshold cycle
DCIS	Ductal carcinoma in situ
ddH20	Double distilled water
DEG	differentially expressed genes
DEmiRs	differentially expressed miRNAs
DMEM/F12	Dulbecco Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides
DTT	1,4-dithiothreitol; EDTA, (ethylenedinitril0)- tetraacetic acid
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptors
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ErbB2	HER-2, EGFR-2, Human Epidermal growth factor receptor 2
FANC	FA, Fanconi anemia
FC	Fold change
FDR	False Discovery Rate
FFPE	formalin fixed paraffin embedded
FISH	Fluorescent in situ hybridization
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEPAS	Gene Expression Pattern Analysis Suite
GFP	Green fluorescent protein
GSEA	Gene Set Enrichment Analysis
GWAS	Genome-wide association studies
HDAC	Histone deacetylase
HER2	Human Epidermal growth factor Receptor 2
HMEC	Normal mammary epithelial cells
HR	Homologous recombination
HRP	Horseradish peroxidase
Hy3^{TM}	fluorescent dye spectrally equivalent to cyanine 3
IDC	Invasive ductal carcinoma
IGF-IR	Insulin-like growth factor-i receptor
IHC	Immunohistochemistry
IRAK1	Interleukin-1 receptor-associated kinase 1
JNK	c-Jun N-terminal kinases
Ki-67	MKI67, antigen identified by monoclonal antibody Ki-67
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LCIS	Lobular carcinoma in situ
LOH	Loss of heterozygosity
mAbs	Monoclonal antibodies
MAF	Minor Allele Frequency
MAPK	Mitogen-activated protein (MAP) kinases

MCF-7	Mammary carcinoma cells type 7
MDA MB 436	Human breast carcinoma cell line, ATCC reference name
MIAME	Minimum information About a Microarray Experiment
miRNA	microRNA, micro ribonucleic acid
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole
NBS1	Nijmegen breakage syndrome 1
NER	Nucleotide excision repair
NFκB	Transcription nuclear factor kB
NHEJ	Non-homologous end joining
NTC	No template control
p21	Cyclin-dependent kinase inhibitor 1A
PAGE	Polyacrylamide gel electrophoresis
PALB2	partner and co-localizer of BRCA2
PARP	Poly ADP (Adenosine Diphosphate)-Ribose Polymerase
PBS	Phosphate Buffer Solution
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PR	Progesterone receptor
PTEN	phosphatase and tensin homolog
Q RT PCR	Quantitative real-time PCR
R	Correlation coefficient
RAD51C	Recombination protein A 51C, RecA homolog E. coli C
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RPM	Rotations per minute
RT	Reverse transcriptase
RT	Room temperature
SAP	shrimp alkaline phospatase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphisms
SURF1	Surfeit locus protein 1
TDLU	terminal duct lobular units
TMA	Tissue microarray
TNBC	Triple negative breast cancer
TP53	Tumor Protein p53 gene
TRAF2	TNF receptor-associated factor 2
TRAF6	TNF receptor-associated factor 6
TRE	Transcriptional Response Element
TSG	Tumor suppressor genes
VEGF	Vascular endothelial growth factor
WT	Wild type
XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3

PRESENTACIÓN

El cáncer de mama es una enfermedad compleja y heterogénea originada a partir de una proliferación anormal y desorganizada de las células que componen el tejido mamario. El cáncer de mama es la segunda causa de muerte más frecuente por cáncer en todo el mundo en las mujeres y es el cáncer más común en las mujeres. La incidencia de cáncer de mama en países desarrollados es más alta que en los países en desarrollo. También es el cáncer más frecuente en las mujeres españolas; con una tasa de incidencia estandarizada por edad de 51 casos por 100.000 mujeres al año, ocupa una posición intermedia entre Europa Occidental y los países de Europa del Este. Sin embargo, la incidencia de cáncer de mama está aumentando alrededor del 2-3% por año (Polan, 2007). Los cambios en el comportamiento reproductivo y el estilo de vida, junto con la introducción de la terapia de reemplazo hormonal se consideran responsable en parte de esta tendencia (Parkin y Fernández, 2006). Aproximadamente el 77% de los casos de cáncer de mama ocurren en mujeres mayores de 50 años de edad. Existen varios factores bien establecidos que se han asociado con un mayor riesgo de padecer cáncer de mama: historia hereditario de la enfermedad, nuliparidad, menarquía precoz, edad avanzada y antecedentes clínicos de cáncer de mama. Los avances en el diagnostico, tratamiento médico y quirúrgico durante los últimos 4 décadas son responsables de la tendencia decreciente de las tasas de mortalidad, a pesar de no disminuir la incidencia. Sin embargo, en aproximadamente el 30% de las mujeres inicialmente diagnosticadas en estadios tempranos de la enfermedad, la enfermedad progresa a estadios más avanzados o metastatiza, siendo la principal causa de mortalidad relacionada por el cáncer de mama.

La gran mayoría de los casos de cáncer de mama son esporádicos, mientras que el cáncer de mama hereditario representa sólo el 5-10% de los casos. Las mutaciones en dos genes de alta susceptibilidad, *BRCA1* y *BRCA2*, explican aproximadamente el 25% de todos los cánceres de mama hereditario, y las mujeres que portan mutaciones en estos genes tienen un riesgo de desarrollar cáncer de mama a lo largo de su vida que es aproximadamente 10 veces mayor que el de la población general. Otros genes de susceptibilidad a cáncer de mama incluyen *TP53* y *PTEN* - asociados con síndromes de cáncer poco frecuentes, mutaciones en el *CHEK2*, *NBS1*, *RAD51C*, *BRIP1* y *PALB2* que están asociados con la duplicación de los riesgos de cáncer de mama. Las mutaciones en estos genes representan un pequeño porcentaje y junto con recién identificados variantes de baja susceptibilidad no explican más de 10% de los casos de cáncer de mama que se producen en las familias que no llevan mutaciones en los genes *BRCA1* o *BRCA2*. Por tanto existe un grupo mayoritario de cáncer de mama hereditario en el que la susceptibilidad genética no puede ser atribuida a mutaciones en los genes estudiados hasta el momento, este grupo se conoce como BRCAX.

Los microRNAs son un grupo de RNAs de cadena sencilla de pequeño tamaño (aprox. 22 nt) que no codifican para proteínas, sino que intervienen en la regulación de RNAs mensajeros. Los microRNAs son sintetizados inicialmente como transcritos primarios que tienen una estructura en forma de bucle con una horquilla formada por bases complementarias (pri-microRNAs). Estos son procesados luego en el núcleo por la enzima Drosha y DGCR8 que corta parte de la secuencia formando los pre-microRNAs. Los pre-

microRNAs se exportan al citoplasma donde son de nuevo procesados para formar RNAs muy cortos de doble cadena por la enzima Dicer. Estos se unen al complejo RISC que separa las dos cadenas, formando los microRNAs maduros. Actualmente hay recogidos mas de mil miRNAs humanos en la última versión de la base de datos miRBase v.18.0 . A pesar de que el número de miRNAs ha ido aumentando rápidamente y probablemente siga haciéndolo, para la mayoría de los miRNAs descritos quedan aún por identificar cuáles son los genes diana a los que regulan.

El principal mecanismo de acción descrito de los miRNAs es mediante la unión de los miRNAs maduros al extremo 3' no codificante de los mRNAs a los que regula, lo cual resulta en la represión transcripcional o degradación de RNA mensajero de estos genes. En cualquier caso, el resultado es la disminución de la expresión de la proteína codificada por el gen regulado por miRNAs. Está descrito que cada miRNA puede regular múltiples genes, así como un mismo gen puede verse regulado por diferentes miRNA. Aunque la función de los miRNAs aun no está bien conocida, parecen ser importantes para un gran número de procesos biológicos, como diferenciación, regulación de la proliferación celular o apoptosis, existiendo numerosos trabajos sobre el papel de los miRNAs en cáncer. La mayoría de los estudios de los miRNAs en tumores se han basado en la comparación de la expresión que estos muestran en tumores y en muestras normales. Algunos miRNAs se sobreexpresan en determinados tipos tumorales y tienen potencial oncogénico, mientras que otros parecen actuar como supresores de tumores y están ausentes o reprimidos.

En cuanto al papel de los miRNAs en tumores de mama, hasta este momento se han realizado diversos estudios de expresión de miRNAs tanto en líneas celulares como en tejidos normales y tumorales de cáncer de mama. Los perfiles de expresión de miRNAs en cáncer de mama esporádico han permitido identificar miRNAs importantes en el desarrollo de estos tumores, que difieren significativamente en muestras tumorales y normales, como mir-10b, mir-125b, mir-145, que se encuentran reprimidos o el mir-21 o mir-155 que están consistentemente sobreexpresados. Además se ha encontrado que los cambios de expresión de miRNAs parecen definir, de forma similar a lo encontrado con los perfiles de expresión de genes codificantes, los diferentes subtipos histológicos (lobulares/ductales, ER+/ER-) y moleculares (luminar A, luminar B, basal-like, HER2+) descritos hasta el momento (13). Sin embargo, es muy poco lo que se sabe del papel de los miRNA en cáncer de mama hereditario.

INTRODUCTION

1. CANCER

Cancer is a genetic disease that arises from an accumulation of mutations in critical genes. It is defined as a malignant neoplasia (tumor), characterized by an abnormal growth and unregulated proliferation of cells that tend to invade surrounding tissues and metastasize to distant sites. Although cancer encompasses many different tumor types, classified by their cellular origin, they all share common hallmarks which comprise a number of biological capabilities acquired during the multistep development of human tumors. These common traits include sustained proliferative signaling, evasion of growth suppressors, resistance to apoptotic signals, replicative immortality, sustained angiogenesis, reprogrammed energy metabolism, evasion of immune destruction, genomic instability and increased mutagenesis, inflammation, invasiveness and ability to form metastasis (Hanahan and Weinberg, 2011).

2. BREAST CANCER

2.1. Mammary gland and biology of breast cancer

Human breasts (mammary glands) are reproductive organs specialized for milk production. Each mammary gland is divided into 15 to 20 lobes, each containing many smaller lobules. The basic units of the mammary gland are the terminal duct lobular units (TDLUs) that coalesce forming a single large duct. TDLUs produce the fatty breast milk that flows, *post-partum* from the lobules through ducts to the areola. Fat and connective tissue fill the spaces between the lobules and ducts, and together with the lymphatic tissue make the mammary gland (**Figure 1A**). Ductal mammary epithelium is comprised of several cell lineages, including secretory luminal epithelial cells and contractile myoepithelial basal cells that reside on basal membrane. The mammary epithelium is maintained by the presence of multipotent mammary stem cells. Mammary epithelial cells organize into three-dimensional structures, which are strongly dependent on a polarized morphology, specialized cell–cell contacts and specific attachments to an underlying basement membrane (**Figure 1B**).

Breast cancer is a complex disease resulting from abnormal and disorganized proliferation of cells that compose breast tissue. About 95% of malignant breast tumors are carcinomas, which originate from the epithelium of the mammary gland. Neoplastic transformation of a carcinoma typically proceeds from a benign, well-differentiated localized tumor, carcinoma *in situ*, to invasive cancer that penetrates basal membrane infiltrating the surrounding tissue, and ultimately to metastatic tumor that disseminates to other parts of the body through lymphatic and blood vessels.

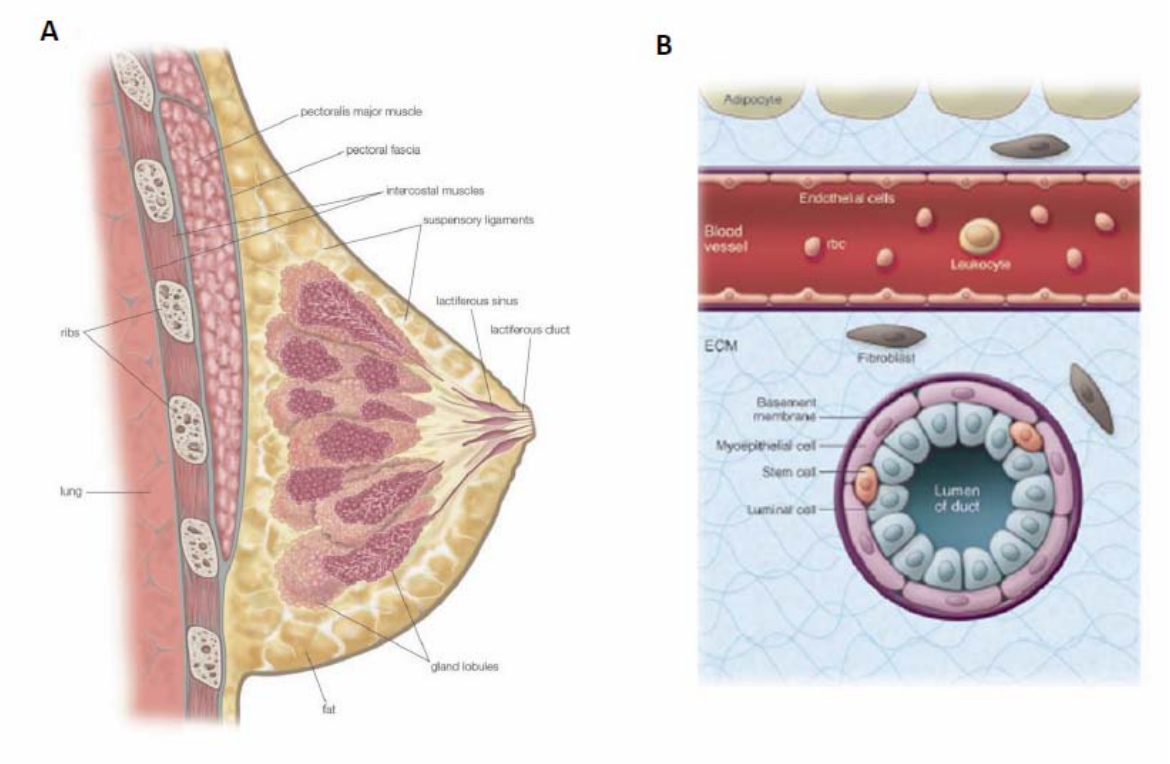


Figure 1. **A)** The anatomy of the breast: sagittal section of the mammary gland. Adapted from ©Encyclopaedia Britannica 2010; **B)** Schematic depiction of normal breast epithelium and surrounding stroma. Adapted from Bertos et al, 2011.

2.2. Breast cancer heterogeneity

2.2.1. Morphological and histological subtypes

Breast carcinomas exhibit a wide range of morphological phenotypes and specific histopathological types have particular prognostic or clinical characteristics. The vast majority of breast tumors are adenocarcinomas originating from mammary epithelium (Cancer.gov, Breast cancer Facts & Figures 2011-2012). Traditionally, breast cancer is classified by pathologists on it histological appearance together with criteria such as clinical features and anatomic sites. In terms of their anatomic localization, breast tumors are classified as in situ carcinomas, confined within the ducts or acini, and invasive or infiltrating carcinomas, that break through the basal membrane into surrounding tissues. In terms of their histology, the latest edition of the WHO classification of breast cancers recognizes the existence of at least 17 distinct histological special types. The majority of in situ carcinomas are ductal carcinomas in situ (DCIS) (83%), while lobular carcinomas in situ (LCIS) are much less common. The most common invasive breast tumor types are infiltrating ductal (70%)

and lobular (6%) carcinomas, while the rare histological subtypes such as medullary, mucinous, papillary, tubular, and other special subtypes contribute only a small proportion (Ellis, 2003).

The staging system for breast cancer is based on the TNM (Tumor, Node, Metastasis)-classification and reflects the extent of spread of the cancer when it is first diagnosed. The TNM classification of tumors uses information on tumor size and how far it has spread within the breast and nearby organs (T), lymph node involvement (N), and the presence or absence of distant metastases (spread to distant organs) (M). Once the T, N, and M are determined, a stage of 0, I, II, III, or IV is assigned, with stage 0 being in situ, stage I being early stage invasive cancer, and stage IV being the most advanced. In general, TNM stage is inversely correlated with the prognosis. Furthermore, taking into consideration the grade of the tumor, based on the assessment of tubule formation, nuclear pleomorphism and mitotic index, breast carcinomas can be classified as: well differentiated - grade 1 tumors, moderately differentiated - grade 2 tumors, and poorly differentiated - grade 3 tumors. Tumor grade indicates tumor aggressiveness and has been recognized as an independent prognostic factor. (Oldenburg et al., 2007; Weigelt and Reis-Filho, 2009).

2.2.2. Molecular subtypes

In the past decade great advances in gene expression profiling using microarray technology have led to better understanding of breast tumor heterogeneity. Initial study by Perou and colleagues revealed previously unknown molecular stratification of breast tumors into reproducible molecular subtypes of breast cancer with different biological features, clinical outcomes and responses to therapy. The groups included two ER negative groups: *basal-like*, characterized by expression of cytokeratine-5 and cytokeratine-17; *ErbB2 tumors*, overexpressing ERBB2 (HER2) and related genes; and *normal-breast like* tumors, expressing genes from adipose and other non-epithelial cells. The ER positive tumors, at first classified as a single group, were subsequently subclassified into at least two different groups: *Luminal A* (with high levels of expression of cytokeratine-8 and cytokeratine-18) and in *Luminal B* differing by level of ER expression and higher proliferation rate (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003). The molecular subgroups have been extended to also include a sixth subgroup which has been named the *claudin- low group*, based on its low expression level of tight junction genes (the claudin genes).

Most ER-positive, low-grade tumors may be grouped into a *luminal* subclass, characterized by a high expression level of luminal CKs (CK 8/18/19), ER, PR, BCL2, P27KIP1, a low expression level of TP53 and ERBB2, and a low grade. This subclass groups about 65–75% of breast cancers. *Basal* breast tumors comprise about 15–20% of all breast cancers and are ER- and PR negative, have a low level of luminal CKs, BCL2, P27, HER2 and a high expression level of P53 and of the basal CKs 5/6 and 17. Most of these tumors have a high grade. HER2-overexpressing tumors are generally characterized by a low, if any, expression level of ER, PR, and P53. These subtypes can be distinguished to an extent based on few immunohistochemistry markers

as: *luminal A* (ER+ and/or PR+, HER2-), *luminal B* (ER+ and/or PR+, HER2+), *basal-like* (ER-, PR-, HER2-, CK5/6+ and/or EGFR+), HER2 (ER- and PR-, HER2+) (Abd El-Rehim et al., 2005; Callagy et al., 2003; Gown, 2009; Tang et al., 2009)

There have been great expectations that gene expression profiles may be used as a tool to define the signature of cancer and predict the prognosis and response to treatment, and that this knowledge might also allow for the development of treatment strategies based on an individual's tumor characteristic (Sorlie et al., 2001; Stingl and Caldas, 2007); (Sotiriou et al., 2003)). In fact, basal-like tumors and HER2 positive tumors were associated to short survival and bad prognosis, while *luminal A* subtype had the best prognosis. Without a doubt, identification of a specific breast cancer subtype characterized by amplification and/or over expression of ERBB2 gene (HER2 tyrosine kinase receptor) has lead to development of targeted therapy Herceptin® (trastuzumab) with great clinical success. Furthermore, several prognostic multi-gene classifiers have been developed and some are approved for clinical use, that although do not outperform standard prognostic factors, can complement them in clinical decision making (Rakha and Ellis, 2011).

2.3. Breast cancer epidemiology and risk factors

Breast cancer is the second most common cancer and the leading cause of cancer death in women. The global burden of breast cancer exceeds all other cancers and the incidence rates of breast cancer are increasing (DeSantis et al., 2011). In 2008 it was estimated that worldwide, 1.38 million women were diagnosed with breast cancer, accounting for around a tenth (10.9%) of all new cancers and nearly a quarter (23%) of all female cancer cases. Female breast cancer incidence rates vary considerably, with the highest rates in Western Europe (89.7 per 100,000 women) and the lowest rates in Eastern Africa (19.3 per 100,000 women) (Ferlay, 2008). Spain occupies an intermediate position between Western and Eastern European countries with 51 cases in 100000 women per year; however, the breast cancer incidence is increasing around 2-3% per year (Polan, 2007). In general, incidences rates are high (>80 per 100,000) in developed regions of the world (except Japan) and low (<40 per 100,000) in most of the developing regions. Breast cancer risk typically increases for women who migrate from low to high risk countries suggesting a strong effect for lifestyle and/or environmental factors. The range of mortality rates is much lower (approximately 6-19 per 100,000) because of the more favorable survival of breast cancer in (high-incidence) developed regions due to better education, implementation of better screening programs, and more effective therapy. As a result, breast cancer ranks as the fifth cause of death from cancer overall. Nevertheless it is still the most frequent cause of cancer death in women in both developing and developed regions (Ferlay, 2008).

There are many different factors that can modify the lifetime risk of breast cancer (Cancer Research UK, 2012; Cuzick, 2008). Age is by far the strongest risk factor (after female gender) for breast cancer. It has been estimated that 1 out of 8 women will develop breast cancer during their lifetime. Estimated risk increases exponentially with age above 50 years, with 81% of cases occurring in women aged 50 years and over..

Demographic factors and ethnicity influence the high variability of breast cancer risk. A large part of variation in risk of breast cancer between developed and less developed countries can be explained by the fact that women in developed countries have fewer children on average and a limited duration of breastfeeding. Breast density is another strong independent risk factor for breast cancer. Women with the more dense breasts (less fat and connective tissue component) have almost five times higher risk of breast cancer than women with the least dense breasts. Reproductive factors that increase the risk of breast cancer include long menstrual history (early age of menarche and late age of menopause), nuliparity, more than 30 years of age at first full-term pregnancy, no or short breastfeeding period. The use of oral contraceptives (OCs) increases the risk of breast cancer in current and recent users, but there is no significant excess risk ten or more years after stopping use. Women currently taking hormonal replacement therapy (HRT) have a temporary 66% increased risk of breast cancer compared to non-users, with risk returning to that of a never-user within five years. Non-reproductive lifestyle factors, such as body mass index (BMI), alcohol consumption, physical activity, diet, medical radiation exposure, working night-shifts, have all been associated to modifying the risk of breast cancer.

Previous breast disease is indicative of a 3- to 4-fold higher risk of developing a new cancer in the other breast or in another part of the same breast. Family history of breast cancer suggests increased risk depending on number of affected members. Having one first-degree relative (mother, sister, or daughter) with breast cancer approximately doubles a woman's risk. Having two first-degree relatives increases her risk about 3-fold. However, over 85% of women who have a close relative with breast cancer will never develop the disease, and more than 85% of women with breast cancer have no family history of it. Genetic factors contributing to increased breast cancer risk are conferred by a presence of germline mutations in one of known breast susceptibility genes.

3. HEREDITARY BREAST CANCER

3.1. Breast cancer families

Family history of breast cancer is a strong indicator of an increased risk, and the existence of families with excess breast cancer cases has been recognized since the Roman ages (Broca, 1866); (Le Dran, 1757). A large majority of breast cancer cases are sporadic, usually detected in older patients (>55 years), while approximately 5% of breast cancer cases arise in patients with strong familial aggregation of breast tumors with various affected members throughout several generations. These cases are known as *hereditary* breast cancer cases, and follow a pattern of autosomal dominant inheritance in multiple affected members, have earlier age of onset, and/or present bilateral tumors. In case there is a suggestive family history but the number or the distribution of cancers is not definitive and the age of onset is variable, families are described as having *familial* cancer (Berliner and Fay, 2007).

3.2. Genetic susceptibility to breast cancer

Heritability studies on monozygotic and dizigotic twins clearly indicated the existence of strong genetic component (Lichtenstein et al., 2000; Peto and Mack, 2000), and early segregation analysis favored a highly penetrant autosomal dominant genetic model. This was confirmed when, in the early 1990s, two high susceptibility genes, *BRCA1* at chromosome 17q21, and *BRCA2* at chromosome 13q12, have been identified through linkage studies and positional cloning without any previous knowledge of their biological function (Miki et al., 1994; Wooster et al., 1995). At first, it was thought that mutations in these two genes will explain up to 80% of familial breast cancer cases (Easton et al., 1995; Ford et al., 1995), but subsequently it was shown that they were responsible for only 20-30% of cases depending on a population in question (Serova et al., 1997). In the following years, the quest for novel susceptibility genes through linkage studies was intensified, but has yielded no reproducible results. However, another approach based on mutational screening of candidate genes selected based on their potential functional impact, has lead to identification of several rare susceptibility genes conferring moderate risk, that altogether account for additional 10% of familial breast cancer cases. Failure to identify novel high-susceptibility gene that would explain a significant portion of the remaining cases strengthened the notion that remainder of familial breast cancer follows a polygenic model where various different genes with incomplete penetrance are causative of the disease or that is due to cumulative effect of common low penetrance susceptibility genes. Finally in the last decade, with the advent of new technologies, the latter hypothesis was subject of intense investigation through genome-wide case-control association studies (GWAS) in large international collaborative projects. This strategy led to identification of a number of low-risk common variants that are presumably responsible for additional 10% of remaining familial breast cancer cases.

Current genetic landscape of breast cancer susceptibility consists of two high-risk (>10-fold) susceptibility genes, several rare intermediate-risk (2-4 fold) genes, and a number of common low-penetrance (< 2-fold risk) alleles. Still, approximately 65% of breast cancer cases arising in high-risk breast cancer families are unaccounted for (**Figure 2**). This represents a big “black box” not only in terms of underlying biology of these tumors, but is also a major problem for genetic counseling of these patients and their families.

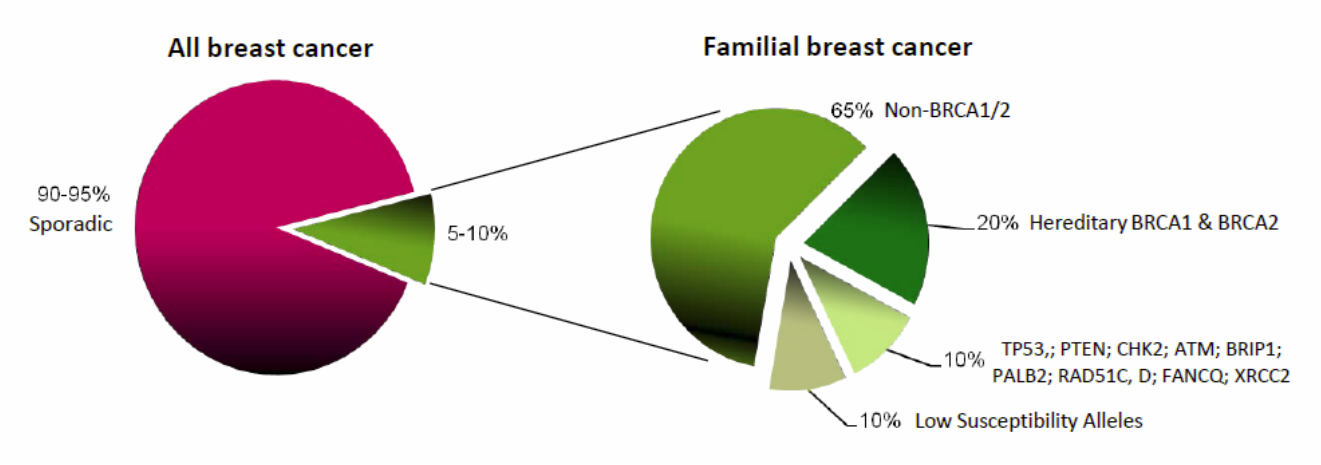


Figure 2. Genetic landscape of breast cancer susceptibility

3.2.1. High-risk breast cancer susceptibility genes BRCA1 and BRCA2

Linkage analysis in large breast cancer family pedigrees lead to mapping of breast cancer susceptibility gene to 17q21 in 1990 (Hall et al., 1990) and in 1994. by positional cloning *BRCA1* gene was identified as first causative breast cancer gene (Miki et al., 1994). Linkage analysis and positional cloning lead to mapping and identification of *BRCA2* in 1994 and 1995, respectively. (Wooster et al., 1995; Wooster et al., 1994) More than 1000 different sequence alterations have been reported in both genes, and novel ones are continuously deposited in the BIC database (Breast Cancer Information Core). Mutations are scattered throughout the large coding regions of the genes, making mutation screening challenging both technically and financially. Vast majority of reported mutations are small insertions or deletions, nonsense mutations, or alterations affecting the splice-site. Many missense substitutions have also been identified, but their role in carcinogenesis is much harder to establish, and therefore, most of them are classified as variants of unknown significance (VUS).

Mutation frequencies in *BRCA1* and *BRCA2* vary between different populations, but are generally very rare with approximate prevalence of 1 in 860, and 1 in 740, respectively (Antoniou et al., 2002) and are are estimated to account for a relatively small percentage (20-25%) of familial risk (Thompson and Easton, 2004). Geographically or culturally isolated ethnic groups, such as Ashkenazi Jewish, Iclandic and Finnish populations, have much higher prevalence of population-specific founder mutations (Abeliovich et al., 1997; Thorlacius et al., 1996). Women with mutations in either *BRCA1* or *BRCA2* have a predicted lifetime risk of breast cancer between 37-85%, and a lifetime risk of ovarian cancer between 15-40% (Antoniou et al., 2003; Ford et al., 1995; Struewing et al., 1997). These estimates vary depending on the study design, patient ascertainment method, population in question, and in addition, additional genetic factors may modify risk in *BRCA1/2* mutation carriers. Furthermore, *BRCA1/2* mutation carriers who have had breast cancer have a 65% risk to develop a second primary breast cancer. Male carriers have four-fold increased risk for prostate cancer and a 100-fold increased risk for breast cancer (6%) compared to that of general population. *BRCA1/2*

alterations also confer increased risk for occurrence of other cancer types including pancreatic, fallopian tube, stomach, colon and prostate cancer for *BRCA1* (Thompson and Easton, 2002), and stomach gallbladder, bille duct, pancreatic, pharynx and prostate cancer for *BRCA2* carriers (van Asperen et al., 2005).

During the past decade many of the cellular and biochemical functions of the BRCA1- and BRCA2-proteins have been discovered characterizing them as tumor suppressors. Both genes are generally considered to be 'caretaker' genes that act as sensors of DNA damage and participate in the repair process. Specifically, both BRCA1 and BRCA2 are necessary for double-stranded DNA breaks by homologous recombination. Their inactivation allows other genetic defects to accumulate and leads to genetic instability. Apart from DNA-repair BRCA1 roles include protein ubiquitylation, chromatin remodeling, cell cycle checkpoint control and transcriptional regulation. Biallelic mutations in BRCA2 (FANCD1) cause a rare type of Fanconi anemia (FA), a recessive syndrome characterized by chromosomal instability, congenital malformations, progressive bone failure, hypersensitivity to DNA crosslinking agents and cancer susceptibility (Taniguchi and D'Andrea, 2006) (Litman et al., 2005). Homozygosity for BRCA1-inactivating mutations, however, results in embryonic lethality, implying the functional differences between the two proteins.

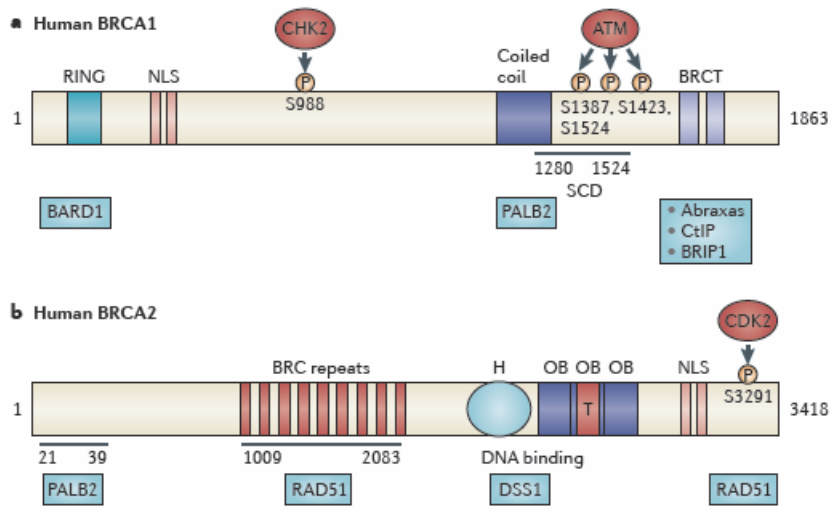


Figure 3. BRCA1 and BRCA2 functional domains. **A)** The BRCA1 N-terminus contains a RING domain that associates with BARD1 and a nuclear localization sequence (NLS). The central region contains a CHK2 phosphorylation site while the C-terminus of BRCA1 contains: a coiled-coil domain that associates with PALB2; a SQ/TQ cluster domain (SCD) that contains ATM phosphorylation sites and a BRCT domain that binds Abraxas, CtIP and BRIP1. **B)** The N-terminus of BRCA2 binds PALB2, and the central region contains BRC repeats that bind RAD51. The BRCA2 DNA-binding domain facilitates binding to both single- and double-stranded DNA. The C terminus contains an NLS and a CDK phosphorylation site that also binds RAD51. Adpated from Roy et al, Nature Reviews Cancer, 2012.

3.2.2. Other rare high-risk breast cancer susceptibility genes

In addition to *BRCA1* and *BRCA2*, mutations in several genes related to rare highly penetrant hereditary cancer syndromes are also associated with increased risk of breast cancer. Germline mutations in the *TP53* gene, which encodes for a protein involved in cell cycle control and apoptosis, are implicated in Li-Fraumeni syndrome, a rare autosomal dominant syndrome with an increased risk of several childhood and adult cancers, including breast cancer (Li and Fraumeni, 1969). Although highly penetrant, the Li-Fraumeni syndrome and germline mutations in *TP53* are very rare, and on overall account for less than 1% of all breast cancer cases (Sidransky et al., 1992). Germline mutations in *PTEN*, a tumor suppressor gene have been identified in families with Cowden syndrome, another rare autosomal dominant cancer syndrome that has been associated with hereditary breast cancer among other cancer types (Liaw et al., 1997). Up to 75% of women with Cowden's syndrome have benign breast disease and the lifetime risk of invasive cancer is estimated to be 25-50% with an average age of diagnosis between 38 and 46 years old (Brownstein et al., 1978). Peutz-Jighers syndrome is an autosomal dominant hereditary cancer syndrome caused by germline mutations in *STK11/LKB1* gene, and is associated with a variety of cancer types including breast cancer (Boardman et al., 1998). It is a very rare syndrome which account for a very small percentage of breast cancer cases, conferring a 29-50% risk by the age of 65 (Lim et al., 2003). In 2010, a novel high-risk susceptibility gene, the Fanconi Anemia (FA) gene *RAD51C*, was identified, showing that a heterozygous mutation in present in approximately 1.5% to 4% of all families predisposed towards breast and ovarian cancer, with high or moderate penetrance (Meindl et al., 2010) . Subsequently several studies in different populations were performed in order to validate the finding. However, some have failed to detect clearly deleterious mutations in *RAD51C* while discarding missense mutations (Clague et al.; Zheng et al.), others have indeed reported highly penetrant mutations albeit at a lower percentage than one described in the initial study (Pelttari et al., 2011) The most recent study, in a large number of breast and/or ovarian families confirmed the existence of both clearly deleterious and functional missense mutations confirmed by functional assays in similar frequencies (1.3%) (Osorio et al., 2012). Like high-risk susceptibility genes *BRCA1* and *BRCA2*, *RAD51C* plays a central role in DNA damage repair as a tumor suppressor gene (Figure 2).

3.2.3. Rare moderate-risk susceptibility genes

Apart from these cancer syndromes, mutations in several other genes have been shown to double the risk of breast cancer. These include rare mutations in *CHECK2*, *ATM*, *NBS1*, *RAD50*, *BRIP1* and *PALB2*.

First report of a truncating mutation, 1100delC in *CHECK2* (Cell Cycle Checkpoint Kinase 2) gene has been identified in a single breast cancer family by candidate gene approach association study, with a reported frequency of 0.5 - 2 % in northern Europe population, conferring an approximately 2-fold increased risk of breast cancer (Meijers-Heijboer et al., 2002). Other *CHEK2* mutations have been subsequently associated with

similarly increased risk of breast cancer in other populations, although reported frequencies reported vary in different populations (Desrichard et al., 2011; Walsh et al., 2006). Germline mutations in *ATM* gene are responsible for the ataxia-telangiectasia (AT) syndrome, a recessive hereditary neurodegenerative disorder that is associated with cerebellar ataxia, telengiectasis, immunodeficiency and genomic instability and increased risk for cancer. However, heterozygous mutation of *ATM* does not lead to the AT phenotype but carriers have a two to five fold risk of breast cancer (Khanna, 2000). A rare protein-truncating mutation in NBS1, first identified in Polish breast cancer patients is associated with approximately 2-fold increased risk. Rare mutations in *BRIP1* (*FANCJ*) were also identified in breast cancer families (Seal et al., 2006) Truncating mutations in *RAD50* were identified in northern Finland conferring a 4-fold increased risk of breast cancer (Heikkinen et al., 2006). And finally, *PALB2* (*FANCN*) truncating mutations have been associated with a 2.3-fold increased risk for breast cancer (Rahman et al., 2007). Of note, biallelic mutations in *PALB2* (*FANCN*) and *BRIP1* (*FANCJ*) cause Fanconi anemia (FA) (Taniguchi and D'Andrea, 2006) (Litman et al., 2005). *BRIP1* encodes a DEAH helicase that interacts with the BRCT domain of BRCA1 and has BRCA1-dependent DNA-repair and checkpoint functions. PALB2 co-localizes with BRCA2, promoting its localization and stability in key nuclear structures, which in turn facilitates BRCA2 functions in DNA repair.

3.2.4. Low-penetrance common variants

In the past two decades more than 1000 reports have been published on associations between candidate genes and risk of breast cancer. However this research area has been problematic because of the many associations that have been published to date, few have been established beyond reasonable doubt. These data were recently comprehensively evaluated in a meta-analysis study linking more than 50 variants in 40 genes to the risk of breast cancer (Zhang et al., 2011). One of the major limitations to the candidate gene case-control association studies is that gene selection is based on the present body of knowledge, which is limited. The development of necessary technology in recent years, along with completion of large scale projects that made whole genome linkage disequilibrium and single nucleotide polymorphism (SNP) data publically available such as International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/) and 1000 genomes project (http://www.1000genomes.org/), enabled the application of agnostic approaches such as genome-wide case control association studies (GWAS) using SNPs as tags. Given that for detection of low risk variants of tens of thousands of cases and control samples are required in order to reach sufficient statistical power, large international consortia and collaborative projects have been formed that facilitated these type of studies.

So far around 20 common alleles have been associated with low risk of breast cancer through GWAS studies in different populations (Fanale et al., 2012; Zhang et al., 2011). All alleles identified are common in general population with minor allele frequencies (MAF) ranging from 0.15 to 0.5, majority of which is conferring less than 1.5-fold relative risk. A major obstacle in the interpretation of these results is that it is not clear if those variants associated are causal SNPs, or merely tags for a potential “real” susceptibility gene

located in the region of linkage disequilibrium proximal to the SNP identified. So far only confirmed association to a functional SNP in *FGFR2* gene has been established through epidemiological and functional studies (Meyer et al., 2008). However, most of other associated SNPs are located in intronic or intergenic regions and the mechanisms by which these variants may influence breast tumorigenesis are largely unknown.

Perhaps the most challenging aspect of these studies is the clinical utility of the variants identified. Their high prevalence in the general population in combination with very low risk, rule out the utility of single variants for breast cancer risk assessment, however, the combined effects may be useful for risk prediction and could facilitate the stratification of population according to associated risk. These women could potentially be candidates for cancer-preventative therapies and increased surveillance, since despite all improvements in the treatment of advanced-stage tumors, early diagnosis and prevention have the most dramatic effect on overall disease-specific outcome. Still, there is a long way to go before this approach could be implemented in the clinics.

3.2.5. Prospects and challenges for identification of novel susceptibility genes

Approximately one third of families with four or five breast cancers occurring in males or females do not carry a mutation in *BRCA1* or *BRCA2* (Ford et al., 1998). On this basis, it is likely that other breast cancer susceptibility genes remain to be discovered. However, there is also an emerging impression that currently unexplained genetic risk in multiple-case families without *BRCA1* or *BRCA2* mutations is unlikely to be due to mutations in a small number of major breast cancer susceptibility genes, but rather represents a heterogeneous mix of polygenic effects and rare mutations in a range of genes that confer high risk in some families (Pharoah et al., 2008). The heterogeneous nature of genetic risk in non-*BRCA1/2* families poses a challenge to the identification of causative genes as evidenced by an absence of clear positional signals emerging from genome wide linkage studies in this group (Smith et al., 2006). In this regard, it is noteworthy that the occurrence of ovarian cancer and male breast cancer in a proportion of multiple case breast cancer families played an important role in the initial discovery of *BRCA1* and *BRCA2* as it focused the search for these genes on families that had an increased chance of shared genetic features on the basis of a common phenotype (Narod and Foulkes, 2004). The absence of other distinctive phenotypes amongst non-BRCA1/2 families have been an impediment to further gene discovery. Perhaps the most comprehensive strategy for the discovery of breast cancer susceptibility genes is the whole genome/exome sequencing of breast cancer families that would capture the complete spectrum of genomic alterations conferring increased risk of breast cancer. This approach is already bearing fruit, with the recent identification of a novel breast cancer susceptibility gene *XRCC2* (Park et al., 2012), not surprisingly involved in homologous recombination DNA repair. However, the major limitation of high-throughput sequencing studies is the data analysis of the vast amount of information obtained, and discrimination of “causative” alterations from “passenger” ones through functional studies and/or mutational screening in large numbers of multiple-case families. Several studies

using this technology are underway and we can expect an explosion in the field in the recent future, hopefully unraveling the architecture of breast cancer susceptibility.

3.3. BRCA1/2 mutation testing and genetic counseling in breast cancer families

So far, the only breast susceptibility genes present in sufficient population frequencies and conferring high enough risk of breast cancer to be eligible for genetic testing are *BRCA1* and *BRCA2*. However, genetic testing for *BRCA1* and *BRCA2* mutations is laborious, complex and expensive, given that these are very large genes without "hot-spot" regions, and last but not least, it is emotionally stressful for the families. Finally, only approximately 30% of families test positive (CNIO, Familial Cancer Unit). It is therefore imperative to identify predictive risk factors and/or biomarkers that associate with a positive mutation status so that the screening could be directed to individuals and families with a high likelihood of carrying a mutation.

Identifying breast cancer patients at increased risk for carrying a mutation in the *BRCA1* and *BRCA2* genes is an important objective in clinical practice. Although age at diagnosis, family history of breast and/or ovarian cancer, and ethnicity are all essential parameters to consider when assessing risk, there are limitations as to how well such factors accurately predict *BRCA1/2* status, even when quantitative risk models are applied. According to Spanish Society for Medical Oncology the criteria for referral of high-risk breast cancer families to undergo genetic testing for *BRCA* mutations are: at least one case of breast cancer diagnosed before 40 years of age; breast and ovarian cancer diagnosed in the same patient; two or more breast cancer cases, one of which is bilateral or diagnosed before 50y of age; breast cancer diagnosed before 50 years of age or bilateral and an ovarian cancer in a first degree relative (FDR) or second degree relative (SDR); tree or more cases of breast and/or ovarian cancer in FDR or SDR; a history of breast cancer in a male relative and one case of breast and/or ovarian cancer in a FDR or SDR; Ashkenazi Jewish heritage with any FDR (or any two SDR) with breast or ovarian cancer; and TN subtype of breast cancer in young patients (<35 y).

Several models that estimate the probability of carrying a *BRCA* mutation have been developed that rely on family history and patients' characteristics. Although these algorithms have acceptable levels of sensitivity, their specificity is clearly suboptimal (Farshid et al., 2006). There are data to suggest that based on the current algorithms for genetic testing, 20% of routine patients attending a multidisciplinary breast cancer clinic would have a probability sufficiently high by at least one algorithm to be offered genetic testing (Shannon et al., 2002). Furthermore, population-based studies have suggested that the reliance of the current genetic algorithms on family history may also be problematic, as up to 9.5% of women with *BRCA1* or *BRCA2* mutations and breast cancer diagnosed before the age of 50 do not have any obvious history of familial early-onset breast cancer or familial ovarian cancer (Frank et al., 2002).

Developing an effective approach to the identification of high-risk individuals is the key to preventing and/or providing early diagnosis of cancer in this patient population. Women carrying mutation in *BRCA*

genes are candidates for additional risk reduction measures such as intensive screening, prophylactic surgery or chemoprevention. The benefits of genetic analysis are, besides improving management in individuals with identified mutation, that the family members that test negative can be released from the greater-than-average cancer risk. Without a doubt, the time and effort expended for a hereditary cancer syndrome diagnosis may significantly reduce both morbidity and mortality in breast cancer.

3.4. Characterization of familial breast tumors

3.4.1. Histopathology

Breast tumors arising in women carrying a *BRCA1* or *BRCA2* mutation have distinct histopathological features and also differ from age-matched sporadic and BRCAX tumors (Lakhani, 1999; Lakhani et al., 1998). BRCA1 tumors are often poorly differentiated ductal carcinomas (grade 3) with higher mitotic count, greater degree of nuclear pleomorphism and less tubule formation than age-matched sporadic tumors, and have higher incidence of medullary carcinoma than BRCA2 tumors or sporadic cases. (Lakhani et al., 2000; Vargas et al., 2011). BRCA1 tumors more frequently have a prominent lymphocytic infiltrate, foci of necrosis and pushing margins. Interestingly, breast tumors associated with *BRCA1* hypermethylation are histopathologically similar to those that are caused by germline mutations in *BRCA1*, in that they are high grade, infiltrating ductal breast cancers that do not express ER (Esteller et al., 2000). Contrasting with BRCA1 tumors, BRCA2 and BRCAX tumors are more heterogeneous and express an extended phenotype spectrum that is closer to that exhibited by sporadic tumors. However, BRCA2 tumors are more frequently moderately or poorly differentiated carcinomas (grade 2 and 3) than age-matched controls, but this has been attributed mainly to a decreased tubule formation, while no difference is generally seen for mitotic count and nuclear polymorphism (Lakhani et al., 2000; Vargas et al., 2011). BRCAX tumors are generally of lower grade (27-50% are grade 1) than BRCA1/2 and even sporadic breast tumors, show more tubule formation, lower mitotic index and less pleomorphisam, and have excess of lobular carcinomas in comparison to BRCA1/2 and sporadic cases. (Da Silva and Lakhani, 2010; Lakhani et al., 2000).

3.4.2. Gene expression profiles

To date, there have been only a handful of studies aimed to identify gene signatures that could be specific to BRCA1, BRCA2, or BRCAX hereditary breast tumors and the number of samples analyzed was generally low due to limitations in acquisition of the necessary fresh tumor material. In the seminal study by Hedenfalk et al. primary tumors from seven carriers of a *BRCA1* mutation, eight carriers of a *BRCA2* mutation, and seven patients with sporadic cases of breast cancer were analyzed by gene expression profiling. Gene expression profiles of BRCA1, BRCA2, and sporadic tumors differed significantly from each other, and set of 176 genes was identified that could distinguish the BRCA1 genotype from the BRCA2 genotype. Genes involved in DNA repair and apoptosis that participate in the activation of cellular responses to stress were more highly

expressed in BRCA1 tumors, as compared to BRCA2 (Hedenfalk et al., 2001). Tumors from BRCA1 mutation carriers have been shown to be similar to the basal subtype of cancers (Sorlie et al., 2003). In a small series of 16 BRCAX tumors, gene expression profiling identified at least two classes, and differentiated them from BRCA1 and BRCA2 tumors. (Hedenfalk et al., 2003). In the study by Fernandez-Ramires et al gene expression profiling of 14 BRCA1 tumors showed segregation mediated by ER status where ER negative tumors sowed overrepresentation of genes involved in the immune response, mostly NFκB-related genes and cell cycle genes (Fernandez-Ramires et al., 2009). Bene et al have shown that BRCA2-associated tumors express genes involved in cell adhesion, and extra-cellular matrix remodeling with activation of MAPK signaling pathway (Bane et al., 2009). Analysis of gene expression in small series of BRCAX tumors confirmed the heterogeneity of this group, demonstrating that they can be stratified into at least 2 groups (BRCAXa and BRCAXb) that can be classified as luminal A and luminal B. BRCAXa group showed additional pathway alterations relating them to BRCA1 tumors (Fernandez-Ramires et al., 2010).

3.4.3. Immunohistochemical features

Many studies have shown that BRCA1 tumors are more often negative for ER (73-90%), PR (80%) and HER2 (0-3.7%) expression, TP53 mutated and positive for cytokeratin 5/6 compared with sporadic tumors and familial non-BRCA1/2 tumors (BRCAX). Based on their characteristics, most BRCA1 tumors are to be classified in the basal subtype. On the other hand, tumors arising in BRCA2 mutation carriers do not differ from controls with regard to ER and PR expression. Thus, ER and PR expression has been reported in around 65% and 40–60% of BRCA2 tumors, respectively. BRCA1 tumors show high proliferative index, evaluated by Ki-67 and overexpression of protein that promote cell cycle progression, such as cyclins E, A or B1. Interestingly cyclin D1 expression is downregulated in BRCA1 tumors in comparison to sporadic, which can be explained by its regulation by estrogen and progesterone. Also BRCA1 tumors underexpress proteins related to the inhibition of cyclin-CDK complexes (p16, p27 and p21). In BRCA2 tumors, the expression of proteins related to cell cycle is similar to that observed in sporadic ER- tumors. BRCA2 tumors show frequently *CCND1* amplification unlike BRCA1 tumors and overexpress apoptotic markers, BCL2 and BAX BRCAX -related tumors are significantly more often positive for BCL2 compared with BRCA1- and BRCA2-related tumors.. A greater incidence of TP53 mutations (23-62%) has been found in BRCA2 tumors compared to sporadic breast cancer. It appears to be difficult to distinguish BRCAX tumors from sporadic tumors and BRCA2 tumors. BRCAX tumors are more frequently ER+ (73-75%), PR- (54-67%) and BCL2 positive (55%) but TP53 negative (78-96%). In this respect they clearly differ from BRCA1 tumors, but are not significantly different from BRCA2 tumors. BRCAX tumors are less frequently P53 and ERBB2-positive than sporadic tumors. Differences among studies can be partly explained by different selection criteria for the BRCAX group, the use of slightly different antibodies or the number of different antibodies used. However, it can also reflect the extensive heterogeneity in the BRCAX group. Honrado et al demonstrated that BRCAX tumors may be classified on the basis of 25 immunohistochemical markers into the five main molecular subgroups

previously identified by expression profiling analysis (luminal A, luminal B, basal-like, normal breast-like and HER2), in addition they can be stratified into high grade ER- tumors expressing proteins related to proliferation and cell cycle progression, and low grade ER+ tumors overexpressing some cyclin-CDK complex inhibitors, antiapoptotic and luminal proteins. (Honrado et al., 2006; Honrado et al., 2005; Palacios et al., 2003; Palacios et al., 2008; Vargas et al., 2011)

3.4.4. Copy number variation and LOH

Specific somatic genetic aberrations determined by metaphase comparative genomic hybridization (CGH) analysis on chromosome 3p (losses), 3q (gain) and 5q (losses) could distinguish BRCA1 related tumors from control tumors with a sensitivity of 96% and a specificity of 76% (Wessels et al., 2002). However, metaphase CGH analysis could not reliably distinguish between BRCA2-associated breast tumors and control tumors or BRCA1-associated breast tumors (van Beers et al., 2005). Based on array CGH analysis which has a higher resolution 169 significant BAC clones were identified which enabled discrimination between BRCA1, BRCA2 and sporadic tumors to some degree. Using hierarchical clustering methods, BRCA1-associated tumors were tightly clustered and separated from sporadic cases, whereas BRCA2-tumors showed a somewhat higher similarity with the sporadic cases, although they still displayed a genomic profile of their own (30% of BRCA2-tumors clustered within the control or BRCA1-group) (Jonsson et al., 2005). All studies showed that BRCA1- associated tumors have the highest frequency of copy number alterations. In familial non-BRCA1/2 associated tumors a significant higher incidence of 8q-gains, 19p-gains, 19q-gains and 8p-losses was observed compared to sporadic tumors.(Gronwald et al., 2005). The study of LOH in 100 BRCAX tumors found LOH frequencies higher than 40% at 1q41, 4p16, 11q23.3, 16p13,16q24, 17p12,21q22,22q11 and 22q13, with the highest frequency at 22q13 that was also specifically lost in BRCAX tumors.

3.4.5. Methylation profiles

A recent study on genome wide methylation profiles in 33 familial breast tumors (11 BRCA1, 8 BRCA2 and 14 BRCAX) demonstrated that different germline mutations can lead to different epigenetic profiles in breast tumors. Tumors with germline *BRCA1* mutation exhibited specific methylation profile, while BRCA2 and BRCAX tumors were mixed in the unsupervised cluster. Methylation profiling did not predict intrinsic subtypes defined by gene-expression profiling in the same sample set, however it predicted mutation status with greater accuracy (64%) than did the gene expression data (29%). Furthermore, BRCAX tumors were subdivided into 2 major classes based on their methylation profiles not correlating with intrinsic subtypes determined by gene expression profiling (Flanagan et al., 2010). Another study on DNA methylation of selected genes was quantified in 99 familial breast tumors with known BRCA1 or BRCA2 mutations formalin fixed, paraffin embedded primary tumors, showing that methylation status was correlated to distinct

tumor characteristics at diagnosis including ER status (+/-), HER2 status (+/-), nodal involvement (+/-) and tumor stage, as well as important clinical outcomes at follow up including cancer recurrence (yes versus no) and the development of distant metastases (Swift-Scanlan et al., 2011).

4. MICRORNAS AS NEW CLASS OF GENE EXPRESSION REGULATORS

4.1. microRNA biogenesis and function

The discovery of the first miRNA lin-4 in *Caenorabditis elegans* in 1993 (Lee et al., 1993) has changed once again our notions of gene regulation adding a new level of complexity in cell signaling pathways and marked a new era in cancer research. MicroRNAs are an abundant class of small ~22nt long single-stranded non-coding RNA molecules. The biogenesis of miRNA involves a complex protein system (**Figure 3**), including Pol II-dependant transcription into a long single stranded pre-miRNA, followed by processing by RNase III complex Drosha/DGCR8 into a short hairpin RNA which is then exported from the nucleus by RanGTP/Exportin5. Once in the cytoplasm it is processed by another RNase III Dicer/TRB protein complex, into a double stranded short RNA and subsequently incorporated into RNA-induced Silencing Complex (RISK) formed by members of the Argonaute family where one of the stands is preferentially incorporated while other is degraded giving rise to a functional RNA-induces silencing complex (RISC) (Kim, 2005). miRNAs act as negative regulators at post-transcriptional level by binding at the 3' untranslated regions (3'UTRs) of their mRNA-targets. Depending on the level of complementarity between miRNA "seed" sequence and its target, they trigger either translational repression, or mRNA degradation (He and Hannon, 2004). Several reports also demonstrated existence of functional miR target sites in coding regions and 5'UTRs, effects of miRs upon transcription and chromatin structure, and of up-regulation of mRNA translation. The exact mechanisms of miRNA function are still far from being fully understood and are a matter of active research. With more than 1500 reported human miRNAs (miRBase 18.0 release, Nov2011) they represent one of the largest classes of gene regulators, accounting for 1-5% of all expressed human genes, and it is estimated that they are regulating two thirds of the protein coding genes (Friedman et al., 2009).

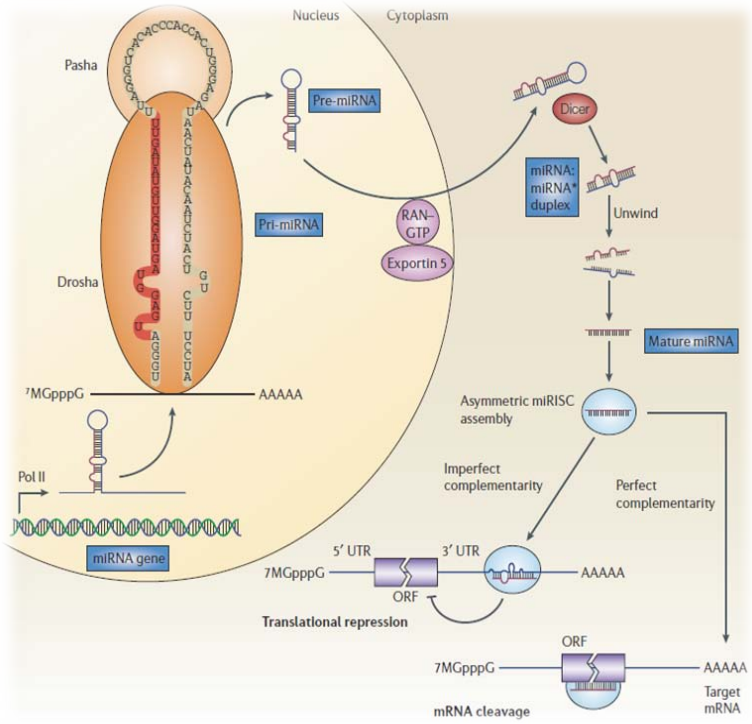


Figure 3. miRNA biogenesis and function. Adapted from Esquela-Kersher, 2006

4.2. MicroRNA expression deregulation in cancer

There is a growing body of evidence demonstrating that miRNAs are involved in crucial biological processes including development, differentiation, apoptosis and proliferation (Calin et al., 2005). Impaired miRNA expression has been extensively implicated in tumorigenesis (Calin et al., 2004a). Abnormal expression of miRNAs has been found in both solid and hematopoietic tumors by various genome-wide miRNA expression analysis techniques (Liu et al., 2004); (Volinia et al., 2006)). miRNAs have been proposed to contribute to oncogenesis since they can function either as *tumors suppressors* (as is the case for miR-15a and miR-16-1) or *oncogenes* (as is the case for miR-155 or members of the miR-17–92 cluster) (O'Donnell et al., 2005). The downregulation or deletion of a miRNA that targets an oncogene leads to tumor formation, and *vice versa*, the amplification or over-expression of a miRNA that targets a tumor suppressor results in tumorigenesis (Calin et al., 2002; Cimmino et al., 2005). Recently, miRNA expression profiling calls a great attention to define various types of cancers. miRNAs seem to classify tumors of different origin more robustly than mRNA based expression profiling (Calin and Croce, 2006; Liu et al., 2004). Furthermore, miRNAs have one great practical advantage over mRNA; they are relatively well preserved in formalin-fixed paraffin-embedded tissues presumably due to their small size and possibly a sheltered micro-environment (Hasemeier et al., 2008)

4.3. MicroRNAs in breast cancer

Specifically, in breast cancer, several studies have identified aberrantly expressed miRNAs in sporadic breast tumors in comparison to normal breast tissue. The seminal study of miRNA expression in 86 breast tumors and normal tissues identified numerous miRNAs deregulated in breast cancer, defined a signature discriminating between normal and malignant breast tissues (Iorio et al., 2005). Ever since a growing number of studies (Farazi et al., 2011; Fassan et al., 2009; Persson et al., 2011; Sempere et al., 2007; Volinia et al., 2006; Volinia et al., 2012) based on different technologies have reported on miRNAs deregulated in breast cancer (**Table 1**). In spite of certain discrepancies in the signatures, there are several recurring miRNAs (e.g. let-7a, miR-125a, miR-125b, miR-143, miR-145, miR-100, miR-10b, miR-101, miR-205, miR-210, miR-29b, miR-497, miR-99a, miR-99b) demonstrating a remarkable reproducibility of miRNA deregulation in sporadic breast cancer. For many of these miRNAs constantly deregulated in breast tissue, important biological functions and molecular targets have been experimentally determined; these are comprehensively reviewed in (O'Day and Lal, 2011). Indeed, many of them, such as miR-21, miR-155, miR-145 and let-7 family are deregulated in multiple types of cancer, including colon, lung and liver cancers (Lujambio and Lowe, 2012).

In addition, miRNA expression was correlated with histopathological features such as estrogen receptor and progesterone receptor status (miR-30) and tumor stage (miR-213 and miR-203). The differential expression of several let-7 isoforms was associated with histopathological features including progesterone receptor status (let-7c), lymph node metastasis (let-7f-1, let-7a-3, and let-7a-2), or high proliferation index (let-7c and let-7d) in breast tumor samples (Iorio et al., 2005). Mattie et al. (Mattie et al., 2006) identified unique sets of miRNAs associated with breast cancers currently defined by their HER2 status or their ER/PR status. Significantly, there was overlap between the miRNAs identified in these profiles and Iorio's panel. Many other studies have identified miRNAs related to specific biological processes and features altered in breast cancer. For example, miR-206, miR-221 and miR-222 were identified as regulators of ER expression, and were find to be overexpressed in ER-negative tumors (Foekens et al., 2008; Zhao et al., 2008).Others such as, miR-335 and miR-126 were associated with increased metastatic potential, and their expression were lost in patient with recurrent disease (Tavazoie et al., 2008). Furthermore, miRNA signatures seem to define, similarly to what has been found by expression profiling of coding genes, different histological (lobular/ductal, ER+/ER-) and molecular (luminal A, luminal B, basal-like, HER2+) subtypes described so far (Blenkiron et al., 2007).

Finally, miRNAs hold great promise for use as clinical biomarkers. Owing to their small sizes, miRNAs are highly resistant to degradation and can be easily extracted from nearly every cell and tissue type. Indeed, miRNAs have been shown to be well preserved in archived formalin-fixed paraffin-embedded sections up to 10 years old (Li et al., 2007), allowing the retroactive analysis of patient samples. Second, a relatively small number of miRNA allows for a robust classification of human cancers (Lu et al., 2005) and outperforms

mRNA signatures in this respect. Third, miRNA detection requires only a small quantity of total RNA for analysis by qRT-PCR and it can be detected by *in situ* hybridization on routine paraffin sections and has immediate potential to be developed as standardized clinical laboratory test. Moreover, circulating miRNAs can easily be measured in whole blood or serum (Heneghan et al., 2010). Several studies have identified cancer-specific miRNAs elevated in the circulation of cancer patients opening up the possibility of using them as noninvasive biomarkers of disease and therapy response.

However, very little is known about the role of miRNAs in familial breast cancer. miRNA expression analysis of hereditary breast cancer can potentially be used for classification purposes, as well as to expand upon our knowledge of differences between different forms of hereditary breast cancer. Likewise it could provide better understanding of the biology of these tumors. BRCAX tumors exhibit histological and molecular heterogeneity that miRNAs could help decipher.

Table 1. miRNAs deregulated in s breast tumors in comparison to normal breast tissue

miRNA		nº of cases		Method	Reference
up-regulated	down-regulated	tumor	normal		
miR-21	**let-7a**	6		Bead-based flow cytometry; Northern blot	Lu et al, Nature, 2005
miR-213, **miR-210**, miR-206, miR-203, miR-202, miR-196, miR-191, **miR-155**, miR-149, miR-136, **miR-128b**, **miR-122a**, miR-34, **miR-21**, miR-9, let-7i	miR-204, **miR-145**, **miR-143**, **miR-125a**, **miR-125b**, **miR-101**, **miR-10b**, **let-7a**, **let-7d**, let-7f	76	10	miRNA microarray; Northern blot	Iorio et al, Cancer Res, 2005
miR-213, **miR-210**, miR-199b, miR-181a, **miR-155**, miR-146, **miR-122a**, mir-31, miR-29a, **miR-29b**, miR-29c, **mir-21**, miR-17-5p	miR-224, **miR-205**, **miR-125b**, **miR-145**, **miR-140**, **miR-130a**, **miR-100**, miR-30c, miR-16, **miR-10b**, **let-7a**	79		miRNA microarray	Volinia et al, PNAS, 2006
miR-21	miR-451, **miR-145**, **miR-205**, **let-7a**	100		miRNA microarray; Northern blot; ISH	Sempere et al, Cancer Res, 2007
miR-26a, miR-26b, miR-499-3p, miR-607, miR-135b, miR-616, miR-769-5p, miR-330-5p, miR-132, miR-149, miR-557, **miR-29b**, miR-657, miR-483-3p, miR-371-3p, miR-593, miR-596	**miR-145**, miR-92a, miR-99b, miR-214, miR-191, miR-454, **miR-10a**, miR-195, **miR-10b**, **miR-130a**, miR-374a, miR-146b-5p, miR-146a, miR-181c, miR-218, let-7g, miR-15b, **miR-125a**, miR-223, **miR-99a**, **miR-140**, **miR-126**, miR-199b, **miR-100**, miR-199a, **miR-125b**	28*		miRNA microarray	Fassan et al, Breast Cancer Res, 2009
miR-196a*, miR-196a, miR-345*, miR-493, miR-301a*, miR-1250, miR-1268, miR-629*, miR-25*, miR-181b, miR-769-3p, miR-181a*, miR-200a, miR-429, miR-182, miR-183*,**mR-21**, **miR-210**	miR-135a, miR-489, **miR-452**, miR-244*, miR-585, miR-1260, miR-27a*, miR-23a*, miR-223, miR-18a, miR-935, miR-1255b, miR-15b*, miR-215, miR-1249, **miR-10b, miR-125b**, **miR-99a**, miR-26a, miR-105, miR-130a, **miR-125a**, **miR-101**, **miR-143**, **miR-100, miR-145***, miR-218, miR-584, miR-139-5p, miR-218*, **miR-376a***, miR-378*, miR-1179, miR-335, **miR-145**, **miR-140**, **miR-126**, miR-101*, miR-328, **miR-378**, miR-139-5p, miR-106a, miR-144*, **miR-451**, miR-486-5p, miR-551b	5	5	Massively parallel sequencing	Persson et al, Cancer Res, 2011
miR-21, miR-142-3p, miR-1425p	**miR-22**, **miR-125a**, **miR-99a**, **let-7a**, **miR-451**, miR-144, **miR-145**, **miR-143**, **miR-143***, **miR-320**, **miR-378**, **miR-497**, miR-16	168	11	Massively parallel sequencing	Farazi et al, Cancer Res, 2011
miR-361-5p, **miR-21**, miR-374a, miR-96, miR-183, miR-374b, miR-16, miR-142-5p, miR-429, miR-182, miR-15b, miR-106b, miR-200c, miR-107, miR-32, miR-26b, miR-223, miR-30d, **miR-128**, miR-200b, miR-342, miR-340, miR-155, miR-142-3p, miR-29c, miR-20a, **miR-29b**, miR-19a, miR-92a, miR-425, **miR-210**, **miR-221**	let-7b, miR-127-3p, **miR-320**, let-7c, miR-652, **miR-378**, **miR-143***, **miR-99a**, **miR-497**, **miR-376a-3p**, **miR-145**, miR-574-3p, miR-193b, miR-221, **miR-140-3p**, **miR-100**, **miR-22**, miR-324, miR-423-3p, **miR-145***, **let-7d**, miR-193a-5p, miR-423-5p, miR-28-5p, **miR-125b**, miR-376c, miR-185, **miR-452**, **miR-125a**, miR-451	80	6	Massively parallel sequencing	Volinia et al, PNAS, 2012

*male breast cancer vs. gynecomastia; in bold letters are miRNAs found to be deregulated in at least two studies

OBJECTIVES

Little is known about the role of miRNAs in hereditary breast cancer. In this study we will sought to examine , on one hand, the expression profiles using microarray technology in BRCA1, BRCA2 and BRCAX-type hereditary tumors. This could allow for a better classification, to one obtained by using mRNA microarrays, and could provide better understanding of the biology of these tumors. BRCAX tumors exhibit histological and molecular heterogeneity that miRNAs could help decipher. The identification of key target genes or pathways regulated by significant miRNAs would give as a better understanding of the mechanisms of tumorigenesis. On the other hand, given the importance of BRCA1 deregulation in both hereditary and sporadic breast tumors, we are interested in investigating the effects of BRCA1 expression on global miRNA expression.

Therefore, the specific aims for the elaboration of this theses project were:

1. To explore the effect of BRCA1 mutation on miRNA expression in breast cancer cell lines
    a. To identify differentially expressed miRNAs and mRNAs induced by BRCA1 gene
    b. To integrate expression data to identify biological processes and pathways modulated by BRCA1
    c. To perform functional studies on selected miRNAs and their target genes.

2. To identify differentially expressed miRNAs between normal breast tissue and hereditary tumors.
    a. To determine biological processes and pathways altered in response to differential miRNA expression
    b. To perform functional studies on selected miRNAs and their target genes.

3. To establish the microRNA expression profile for the different subtypes of hereditary breast cancer.
    a. Identify differentially expressed miRNAs between different tumor subgroups, BRCA1, BRCA2 and BRCAX
    b. Analyze the heterogeneity of BRCAX group

MATERIALS AND METHODS

1. PATIENTS AND SAMPLES INCLUDED IN THE STUDY

1.1. Sample proceedings and ethics statement

Breast cancer tissue samples used in this thesis were collected through collaborations with Spanish Hospitals and centers of investigation. The three different studies in this thesis were carried out using two different sample series:

Fresh-frozen (FrFr) breast tissue samples were acquired from Fundacion Jimenez Diaz and Hospital Clinico San Carlos in Madrid, Institut Catala d'Oncologia in Barcelona, Centro de Investifgacion del Cancer from Salamanca and Instituto dei Tumori in Milan. Breast tissues were obtained immediately after surgical excision, snap-frozen and preserved in blocks with OCT (polyvinyl matrix), for subsequent evaluation and section. Percentage of tumor cells in the tissue was estimated by a pathologist reviewing 5 μm sections stained by hematoxiline and eosin. Only tumors samples with more than 80% of tumor cells were analyzed by microarray.

Formalin-fixed paraffin embedded (FFPE) breast tissue samples were acquired through several participating Spanish hospitals and centers: Gregorio Marañon, Hospital San Pablo, Fundacion Jimenez Diaz, Hospital La Paz, H Santa Caterina (Girona), P. de Hierro, H. Severo Ochoa, H. Ramón y Cajal. Normal breast tissues were obtained after breast reduction surgery from healthy individuals with no family history of breast cancer. FFPE tumor tissues were stained by hematoxylin and eosin and examined by the pathologist. Marked the tumoral area was separated into a new block and 3 x 30μm sections were made for subsequent RNA extraction.

Informed written consent was obtained from all individuals involved in this study to perform genetic studies and to use exceeding material for research, and the research project has the approval of the ethics committee of the Spanish National Cancer Research Centre (CNIO), Comité de ética de la investigación y de bienestar animal del Instituto de Salud Carlos III.

1.2. Accrual of familial breast cancer patients

Patients belonging to high-risk breast cancer families and were selected according to Asociacion Española Contra el Cancer (AECC) inclusion criteria: families with at least three females affected with breast cancer; or at least two first-degree females affected with breast cancer (at least one of them diagnosed before 50); or at least one case of female breast cancer and at least one case of either ovarian, female bilateral breast or male breast cancer. All patients have undergone full BRCA1/2 gene testing for

mutations and large rearrangements using standard procedures (Bradbury and Olopade, 2007; Osorio et al., 2000). Individuals with no mutations identified in BRCA1 or BRCA2 genes were designated as BRCAX.

1.3. Samples cohorts included in different studies

During the elaboration of this thesis three different studies were performed including variable number of samples.

First study: thirteen hereditary breast cancer cases harboring BRCA1 mutations, as well as 4 normal breast tissues from formalin fixed paraffin embedded (FFPE) tumor tissue series, were used to check the expression of selected miRNAs.

Second study: a total of 54 fresh-frozen breast tissues were used. Sample series analyzed by microRNA microarray profiling included 22 frozen hereditary breast tumors consisting of 3 BRCA1-mutated, 5 BRCA2 and 14 non-BRCA1/2 (BRCAX) samples, and 14 normal breast tissues including 3 from BRCA1-mutation carriers, 5 from BRCA2-mutation carriers, 1 normal breast tissue from contra-lateral breast of patient with BRCAX-type tumor, and 5 normal breast tissues obtained after breast reduction surgery from healthy individuals with no family history of breast cancer. The tissue collection used for validation included 18 paired fresh frozen samples from sporadic breast tumors and their adjacent normal breast tissue counterparts.

Third study: a series of 80 formalin–fixed paraffin-embedded (FFPE) breast tissues comprised of 66 familial breast primary tumors from 13 BRCA1 mutation carriers, 10 BRCA2 mutation carriers and 43 BRCAX tumors; 10 sporadic breast carcinomas and 6 normal breast tissues obtained after breast reduction surgery from healthy donors with no family history of breast cancer were analyzed by microRNA microarray profiling.

2. BREAST CANCER CELL LINES AND FUNCTIONAL STUDIES

2.1. Breast cancer cell lines

Breast cancer cell lines were used as a model to study functional effects of gene and miRNA expression. Parental HCC 1937, derived from hereditary BRCA1 mutated breast tumor and HCC 1937/BRCA1wt stably expressing full length BRCA1, were kindly provided by Dr. J. Chen (Yale University School of Medicine Department of Therapeutic Radiology, New Haven, USA). Normal Human Mammary Epithelial Cell line (HMEC) was purchased from Clonetics (San Diego, CA). The MDA-MB-436 cell line was kindly provided by Dr. K.S Massey-Brown from Department of

Pharmacology and Toxicology, University of Arizona, Tucson, USA,. MCF-7 breast cancer cell line was obtained from Cancer Epigenetic Group at Spanish National Cancer Centre, Madrid, Spain.

2.2. Maintenance and subculturing of cells

HCC 1937 cell lines were cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10%FBS (Fetal Bovine Serum, Gibco-BRL, Grand Island, NY, USA), while derived HCC 1937/BRCA1w was maintained in DMEM/F12 with 10% FBS. HMEC cells were grown in, Mammary Epithelial Growth Medium - MEGM (Clonetics) supplemented with growth factors SingleQuots (Clonetics) in absence of FBS. Both MCF7 and MDA MB 436 cell lines were cultured in RPMI supplemented with 10% FBS. Breast cancer cell lines were cultured in corresponding media and maintained at 37ºC in 5% CO2. All media were supplemented with fungizone, and penicillin/streptomycin (Gibco-BRL). Cells were passaged at approximately 80-90% confluence.

2.3. miRNA transfections

miRNAs were routinely transfected into cells using Oligofectamine reagent (Invitrogen, San Diego, CA). Pre-miRNA oligonucleotides (pre-miR-146a, pre-miR-99b, pre-miR-205, miR-30c and scramble control) were purchased from Ambion (Austin, Texas, USA). For experiments in 6- well plates, 100 000 cells/well were plated in 2ml of growth media without antibiotics to a density of 50-60%, 24 hours prior to transfection. For experiments in 24-wells approximately 40000 cell/well were seeded in a 500ul of antibiotic-free media to a density of 40%, 24 hours pior to transfection. Stock transfection mixes were made according to manufacturer's instructions. Briefly, 2.5ul of Oligofectamine reagent was diluted in 500ul of Opti-MEM I Medium with L-glutamine (Invitrogen, CA, USA) and incubated 5 minutes at RT. In another tube pre-miRNA was diluted with Opti-MEM to a final concentration of 50nM. Both mixes were incubated together for 30 minutes at RT to allow complex formation between miRNA and lipids. To transfect cells the growth media was removed and replaced with 0.5ml of Opti-MEM media, and 500ul of the appropriate transfection mix. Control cells were treated with a scramble miRNA (Ambion). Cells were incubated at 37^0C for 12h after which time media was replaced with the same volume of fresh full growth media. At 48 hours after transfection cells were harvested for mRNA and/or protein analysis.

2.4. Luciferase reporter assays

2.4.1. Construction of reporter vectors

1.1.1.1. Molecular cloning of pGL3-KRAS 3'UTR vector

Expression vector pGL3-Control from Promega (**Figure 3**) was used for molecular cloning of KRAS gene 3' untranslated region (3'UTR) downstream of Firefly luciferase gene. 3'UTR sequence of the KRAS was retrieved through NCBI nucleotide database. A 300bp fragment of the 3'UTR region of KRAS gene containing miR-30c binding site, was amplified by PCR from human genomic DNA, and cloned into a modified pGL3-Control vector (Promega) at the SacII and EcoRI site, immediately downstream of the luciferase stop codon. Primer sequences used to amplify this region were RAS3UTR-F: 5'CACGAATTCCACACCCCCACAGAGCTAAC3' and RAS3UTR-R: 5'TTCCCGCGGTGTTTGATATGACCAACATTCCT 3'.

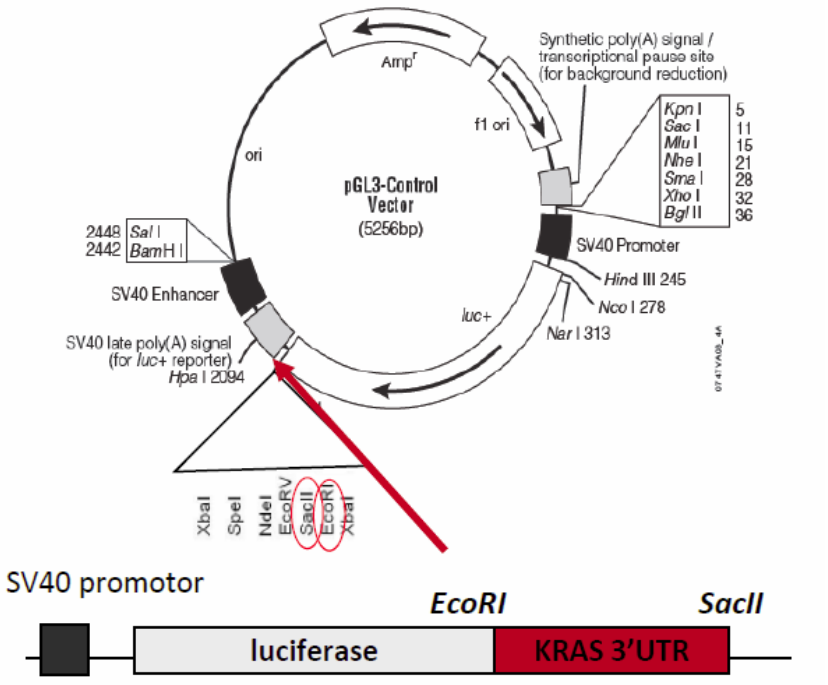


Figure 3. pGL3-Control-Modified cloning vector

1.1.1.2. Reporter vector for TRAF2 3'UTR

Expression vector OmicsLink miRNA Target Clone (GeneCopoeia, Rockville, MD) was purchased from GeneCopoeia (GeneCopoeia, Rockville, MD). The miTarget vector expressed full length TRAF2 3'UTR cloned downstream of Firefly luciferase gene stop codon and Renilla Luciferase gene (**Figure 4**)

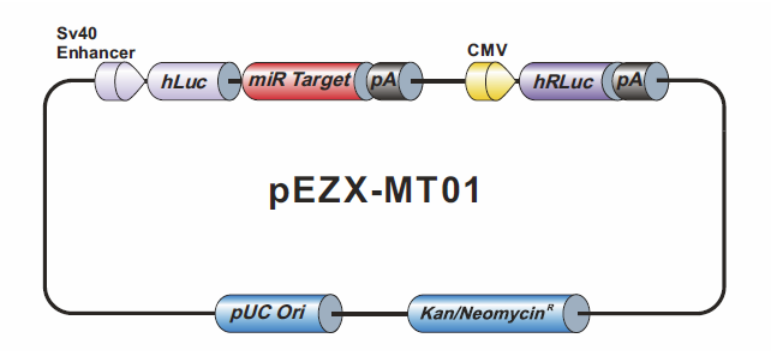


Figure 4. Vector backbone of miTarget miRNA 3' UTR target clones (GeneCopoeia)

The plasmid constructs were propagated by transformation of JM109 *E.Coli* bacterial strain, by adding 3ul of DNA to 20ul of bacteria, incubating 10min on ice, haeat-shocked at 42ºC for 30s and grown in 10ml of LB media (10g/l triptone, 5g/l of yeast extract, 10 g/l of NaCl, pH7.0) at 37ºC overnight with shaking at 300rpm. purified by miniprep (QIAGEN) and used in subsequent luciferase reporter assay. Correct vector construction was verified by restriction digestion and direct sequencing.

2.4.2. Transfections and luciferase reporter assay

To verify direct binding of the miRNA of interest to its target gene we performed target *in vitro* assays using luciferase reporter system (**Figure 5**).

Pre-miRNA oligonucleotides (pre-miR-146a, pre-miR-99b, pre-miR-205 and scramble control) were purchased from Ambion (Austin, Texas, USA). For luciferase reporter target *in vitro* assays 250 ng OmicsLink miRNA Target Clone (GeneCopoeia, Rockville, MD) containing Renilla Luciferase and TRAF2 3'UTR cloned downstream of Firefly luciferase gene, together with 25 pmol (50 nM) of pre-miRNA oligonucleotides, were transfected using Lipofectamine 2000 reagent (Invitrogen, San Diego, CA) into HCC1937 cells in a 24-well plate format following manufacturer's instructions. HCC1937 cells were co-transfected with the TRAF2 3'UTR reporter construct along with each individual pre-miRNA (Ambion), combination of miRNAs, scramble control or mock transfected. Cells were grown for 48h, after which cells were harvested and luciferase activity was assayed with Dual-Luciferase Assay System (Promega) according to manufacturer's instructions. Experiments were performed in triplicate and normalization was performed using Renilla luciferase activity.

To assess miRNA-30c regulation of KRAS gene expression we carried out dual-luciferase reporter assay by co-transfecting MDA-MB-436 cells with 25 pmol of pre-miR-30c or scramble control (Ambion), along with 500 ng of KRAS 3'UTR- firefly luciferase construct and 7.5 ng of Renilla luciferase vector, using Lipofectamine 2000 (Invitrogen) per well, according to the manufacturer's protocol in a 24-well plate format. Cells were grown for 48h, after which luciferase activity was assayed with Dual-Luciferase Assay System (Promega). Experiments were performed in triplicate and Renilla luciferase activity was used for transfection variation normalization.

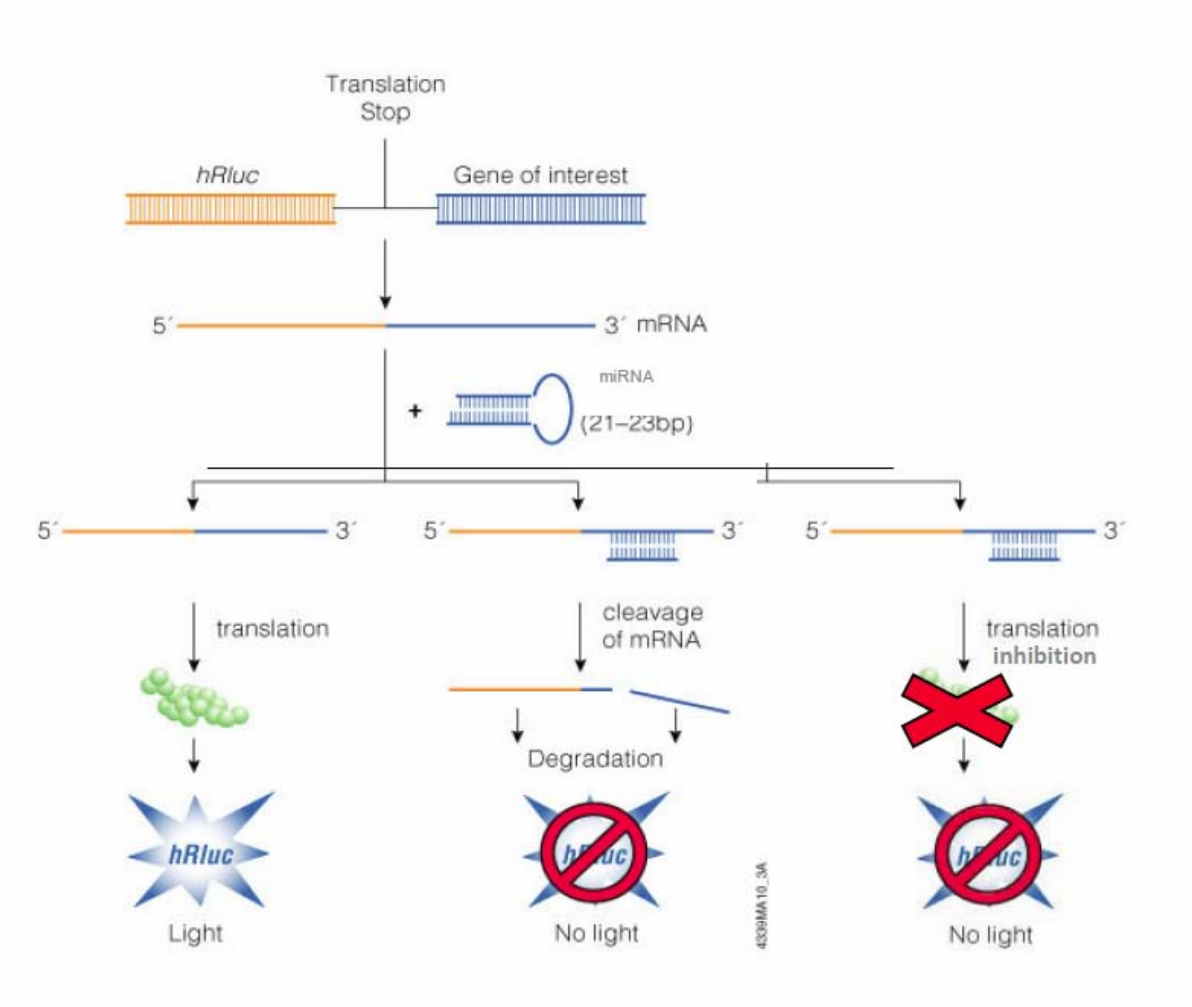


Figure 5. Outline of the luciferase reporter assay. Firefly luciferase gene with 3'UTR region of interest cloned immediately downstream of the stop codon, codes for an oxidative enzyme that converts luciferin substrate into oxiluciferin in a reaction that emits light. Photon emission is detected by luminometer, and the signal intensity is directly proportional to the amount of the enzyme. In the presence of a miRNA that binds to the 3'UTR and induces either mRNA degradation or translational inhibition, production of luciferase enzyme is reduced/abolished resulting in lower signal emission.

2.5. Cell proliferation assays

Cell proliferation rate was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MDA-MB-436 cells were seeded in 24-well plates one day before transfection at 30-40% confluence in antibiotic-free media. Cells were transfected in triplicate independent experiments using Oligofectamine (Invitrogen) with 50 nM of pre-miR-30c/pre-miR-Scramble oligonucleotides or mock transfection control. Cells were incubated in 1µg/µl MTT Formazan (Sigma-Aldrich) diluted in 500 µl normal culture medium at 37ºC for 5h. The assay is based on the cleavage of the yellow MTT tetrazolium salt to purple formazan crystal by metabolicly active cells. The formazan is then solubilized

with DMSO, and the concentration determined by optical density at 570 nm by means of a standard microplate absorbance readers (Bio-Rad, Hercules, CA, USA). Cell viability was determined at 24, 48, 72, 96, 120 or 144 hours after transfection. Each value represents the average of triplicate wells in representative of 3 independent experiments.

2.6. Cell based multi-pathway activity assays

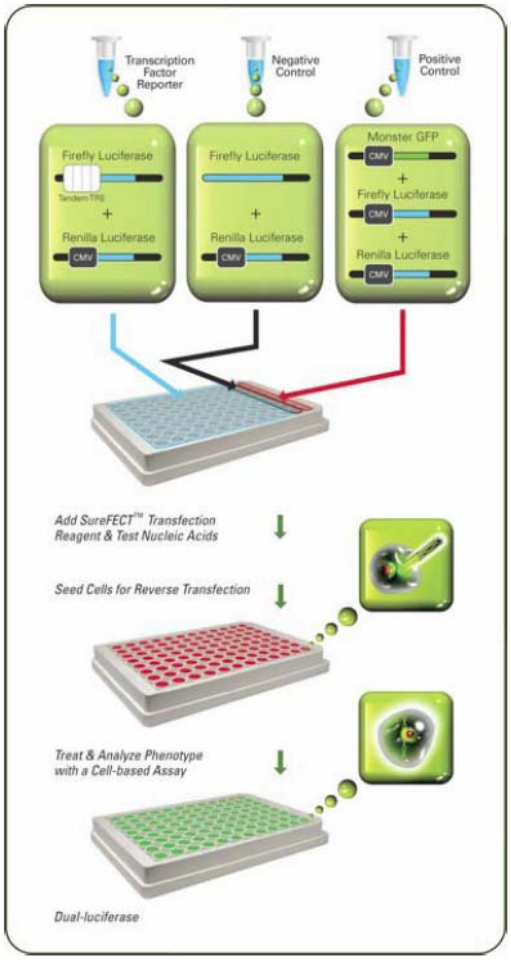
2.6.1. Experimental procedure

Using Cignal Multi-Pathway Reporter Assay Kit (SABiosciences, MD, USA) we evaluated activation of ten different cancer related pathways (**Figure 6**). The HCC1937 and HCC1937/BRCA1 were reverse-transfected with the transcription factor-responsive reporter, negative control, and positive control constructs using SureFECT^{TM} (SABiosciences) according to manufacturer's instructions. The change in the activity of each signaling pathway is determined by comparing the normalized luciferase activities of the reporter in BRCA1-null versus BRCA1-wt expressing HCC1937 cells. For assessment of miRNA effect on pathway activation, 200 pmol of pre-miR-146a, pre-miR-99b, pre-miR-205 or negative control were co-transfected along with pathway reporter in HCC1937 cells. The transfection efficiency was monitored by GFP expression, using fluorescence microscopy. At least three independent transfections were carried out in triplicate for each of the conditions tested with each reporter assay.

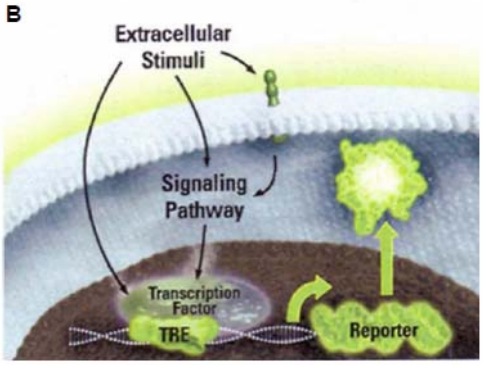
2.6.1. Statistical analysis

Statistical analysis was performed using SPSS version 17.0 (SPSS Inc, Chicago, Illinois). All results are expressed as mean ± standard deviation (SD) of at least three independent experiments. Non-parametric Mann–Whitney U-test and parametric bilateral t-test were used for statistical hypothesis testing. Nominal two-sided p-values less than 0.05 were considered statistically significant.

A



B



C

Pathway	Transcription Factor
Wnt	TCF/LEF
Notch	RBP-Jκ
p53/DNA Damage	p53
TGFβ	SMAD2/3/4
Cell cycle/pRb-E2F	E2F/DP1
NFκB	NFκB
Myc/Max	Myc/Max
Hypoxia	HIF1A
MAPK/ERK	Elk-1/SRF
MAPK/JNK	AP-1
Negative Control	
Positive Control	

Figure 6. Overview of the Cignal Reporter Assay procedure. The plate format of the Cignal Multi-Pathway Reporter Arrays are delivered in a 96-well cell culture plate **(A)**. Each reporter and control assay is dried down in each column of the plate (8 wells per assay). The modulation of a signal transduction pathway results in a change in the activity of a downstream transcription factor **(B)**. The pathway-focused transcription factor-responsive luciferase reporters consist of a combination of specific transcription factor binding sites and basic promoter elements that drive the expression of a luciferase gene **(C)**. This alteration in transcription factor activity affects its ability to bind a specific DNA target sequence, which in turn changes the expression level of luciferase enzyme. A change in the expression of luciferase enzyme can be easily monitored by a change in luminescence intensity.

3. PROTEIN-BASED ASSAYS

3.1. Protein extraction and quantification

For western blot analysis cells were transfected with 50nM pre-miRNA oligonucleotides as described in chapter 2.3. Cells were grown for 48h after transfection, and harvested for protein extraction. Cells were trypsinized and after 2min resuspended with corresponding growth media with 10% FBS. Cells were collected into 15ml conical tubes, washed with 1x PBS and centrifuged at 1200 rpm for 5 minutes. Cell lysates were prepared by resuspending cell pellets in 40ul RIPA buffer (Sigma-Aldrich) with protease inhibitors (Roche) per 10^6 of cells. Cells were sonicated for 30sec (cycle: 15s sonicate 15s rest) and incubated for 30 minutes at 4ºC, followed by centrifugation at 20000g for 20 minutes at 4ºC. Supernatant was collected and protein concentration was measured by Bio-Rad protein assay (Bio-Rad laboratories) using bovine serum albumin (BSA) (Pierce) to create a standard curve with known concentrations of protein.

3.2. Immunoblotting

Equal amounts of protein (50µg) were separated by SDS-PAGE on 4-12% pre-casted gels NuPAGE Bis-Tris Gel (Invitrogen, Carlsbad, CA) at 120V for 1 hour using the XCell SureLock Mini-Cell (Invitrogen) electrophoresis cell. Proteins separated on the gel were electrotransferred during 1h at 30V to nitrocellulose membrane (Watman) using 1x NuPAGE Transfer buffer (Invitrogen) with 20% methanol. Equal loading of proteins was verified by Ponceau S staining with 0.1% Ponceau red in 5% acetic acid for 2 min. Ponceau S was washed out with 1xTBS with 0.05% Tween detergent and the membranes were blocked with 5% non-fat dry milk in 1xTBS with 0.05% Tween detergent for 1 h at room temperature. The membranes were then washed three times for 5 minutes with 1xTBS with 0.05% Tween detergent and incubated with primary antibody for 1 hour at room temperature. The following primary antibodies were used for protein detection: mouse monoclonal antibody against KRAS (F234, Santa Cruz) at 1/200 dilution, mouse monoclonal antibody against TRAF2 (C-20, Santa Cruz) at 1/500 dilution and mouse polyclonal antibody against GAPDH (CNIO, Monoclonal Antibodies Unit) at 1/50 dilution. Next, membranes were washed three times for 5 minutes with 1xTBS with 0.05% Tween detergent and incubated with the corresponding horseradish peroxidase (HRP) conjugated (Dako, Glostrup, Denmark) secondary antibody at 1/1000 dilution for 1 hour at room temperature. The antibody visualization was carried out with enhanced chemiluminescence (ECL) Detection System (Dako) and BiomaxLight membranes (Kodak). Membranes were scanned and signal was quantified using Image J program. Protein content was determined relative to loading control signal.

3.3. Tissue microarrays

A subset of FFPE samples (41) was included in tissue microarrays (TMA) described previously in (Honrado et al., 2006; Honrado et al., 2005; Palacios et al., 2003). Representative areas of the tumors were selected on hematoxylin and eosin-stained sections and marked on individual paraffin blocks (**Figure 7**). Two tissue cores (1 mm in diameter) were obtained from each specimen. The tissue cores were arrayed into a receptor paraffin block using a tissue microarray workstation (Beecher Instruments, Silver Spring, MD), as described previously (Hardisson 2003). A hematoxylin and eosin-stained section of the array was reviewed to confirm the presence of morphologically representative areas of the original lesions.

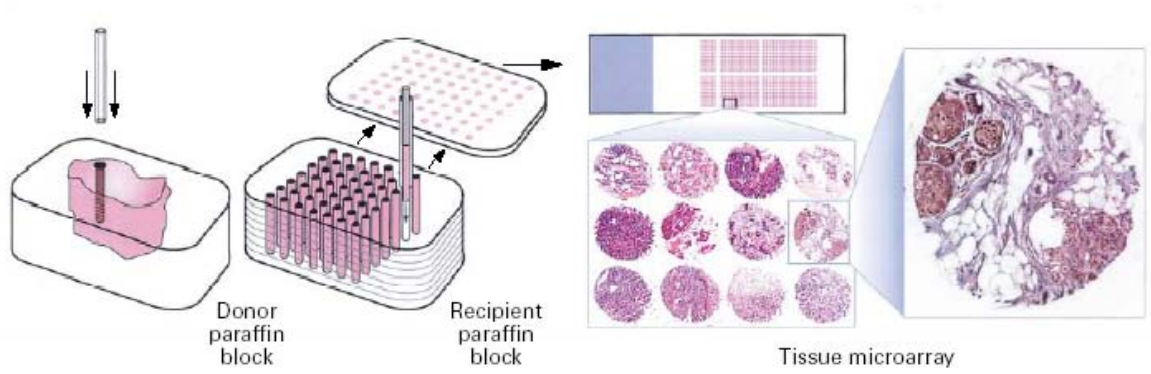


Figure 7. Tissue microarray construction. Adapted from Hedenfalk et al. 2003, PNAS.

3.4. Immunohistochemistry

Imunochistochemical information on tumor grade, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2/ErbB2) gene expression and/or amplification, BRCA1 methylation, Ki-67, p53 and EGFR was available for 41 samples from tissue microarray (TMA) described previously in (Honrado et al., 2006; Honrado et al., 2005; Palacios et al., 2003). For remainder of samples (15) information on tumor grade, ER, PR, HER2/ErbB2 status was obtained from either hospital pathology report when available, or by staining individual tumor sections using same criteria as described in (Honrado et al., 2006).

Immunohistochemical staining on TMA sections was performed by the EnVision method with a heat-induced antigen retrieval step with help of the Immunohistochemistry Unit at CNIO. Staining results were assessed by a pathologist. Tumor grade was assessed based on Bloom-Richardson scoring system. ER and PR were scored based on two-stage scoring system: positive (1) for >10% of ER/PR positive cells and negative (0) for less than 10%, as described previously. Ki-67 expression was

classified according to 3-stage scoring system: 0-5%, 6-25% and >25%. HER2 was called positive either by detection of ERBB2 gene amplification by FISH analysis and/or 3+ staining by DAKO system on HercepTest™. Where duplicate cores gave discordant results, the higher score was used. Tissue cores that failed to adhere to the glass slide were uninterpretable and were excluded. Breast cancer cases were classified into four subtypes based on IHC-model (Tang P. et al, 2009) as: luminal A (ER+ and/or PR+, HER2−); luminal B (ER+ and/or PR+, HER2+); Triple Negative (ER−, PR−, HER2−) and Her2/neu overexpressed/amplified (ER−, PR−, HER2+). Full information on immunohistochemical data in breast cancer samples is represented in **Supplementary Tables 1 and 2**.

3.5. Statistical analysis

The χ2-test and Fisher's exact test were employed for the analysis of the homogeneity of the distribution of the expression of immunohistochemical markers, tumor grade and subtypes between the groups of tumors. Fisher's exact test permits calculation of precise probabilities in situation where sample sizes are small so the normal approximation and χ2-square calculations are liable to be inaccurate. Two-tailed Fisher's exact test was used for pair-wise comparison of markers to detect differences between two groups of tumors. The statistical program SPSS 13.0 (SPSS Inc, Chicago, IL, USA) and R/Bioconductor were used for this analysis.

4. RNA EXPRESSION ANALYSIS

4.1. RNA extraction and quantification

Total RNA was extracted from cell lines and fresh-frozen tissue using Trizol (Invitrogen) according to manufacturer's instructions. RNA from FFPE tissue samples was extracted using miRNeasy FFPE kit (QIAgen) according to the manufacturer's instructions. RNA quantity and quality from FFPE tissues was assessed by NanoDrop ND.1000 UV-VIS-Spectrophotometer version 3.2.1 (Nanodrop Technologies, Wilmington, DE, USA). RNA quantity and quality from fresh-frozen samples was determined using the RNA 6000 Nano Assay kit on an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA). Samples used for mRNA expression microarray analysis had an average RNA integrity number value of 9 and a minimum RNA integrity number value of 7.

4.2. Real time quantitative PCR analysis (qRT-PCR)

4.2.1. Detection of mRNA by q RT-PCR

One microgram of total RNA was reverse transcribed using MMLV Reverse Transcriptase (Invitrogen) and random hexamer primers following manufacturer's instructions. In brief, first template RNA was incubated with 500ug/ml of hexamers at 70°C for 5 minutes, and placed on ice. Subsequently the following components were added in an order: MMLV buffer 1X, dNTPs (Fermentas) (1mM, final conc.), RNAsin (20u) (Promega), 40U of MMLV enzymes (Invitrogen) to reach a final volume of 20ul. The reaction mixture was incubated 2h at 42°C. The cDNAs were subjected to quantitative RT-PCR assay with the use of FAM/NFQ fluorescently labeled probes TaqMan (Roche Universal Probe library, Roche) and TaqMan Universal PCR Maser Mix (PE Applied Biosystems) in an ABI Prism Sequence Detection System 7900HT (Applied Biosystems) using the oligonucleotides and probes listed in Table __. Briefly, the q-RT-PCR set up included 2µl cDNA, 2xMaster Mix, Primer set (20µM) and the TaqMan probe (10µM) in a final volume of 12µl. The amplification conditions consisted of an initial step at 95 ºC for 10 min, followed by 50 cycles of 15 s at 95 ºC and 1 min at 60 ºC. Negative controls were present in all series of PCRs and all assays were carried out in triplicates. Relative expression was calculated using the comparative Ct method. Normalization of the samples was carried out with the internal using ß-actin was used as internal control.

Table 2. Oligonucleotide primers used for mRNA qRT-PCR

Gene name	Primers	Sequence (5'-3')
KRAS	Forward primer:	tggacgaatatgatccaacaat
	Reverse primer	tccctcattgcactgtactcc
	Universal ProbeLibrary	#62
TRAF2	Forward primer:	gcatacccgccatcttctc
	Reverse primer	gcgttcaggtagatacgcagaca
	Universal ProbeLibrary	#11
β actin	Forward primer:	ccaaccgcgagaagatga
	Reverse primer	ccagaggcgtacagggatag
	Universal ProbeLibrary	#64

4.2.2. Detection of microRNAs by qRT-PCR

For miRNAs quantitative RT-PCR analysis was performed using miRCURY LNA™ microRNA PCR System (Exiqon A/S, Vedbaek, Denmark). First, 25ng of total RNA was reverse-transcribed using miRNA-specific primers and First-strand cDNA Kit (Exiqon), and the cDNA was used as a template for the quantitative PCR reaction using miRNA-specific LNA™ PCR primer and Universal PCR primer. Briefly, master mix was prepared consisting of 2µl of miR-specific primers, 0.5 ul of dNTPs, 0.5ul of RNase inhibitor and 0.5ul of reverse transcriptase; 3ul of the master mix were added to 2ul (25ng) of total RNA to a final volume of 5ul. Reaction was incubated at 50ºC for 40 minutes, and heat inactivated at 85ºC for 5 minutes. The cDNA was diluted in 1/10 ratio and used for quantitative PCR reaction. The reaction mix for the qPCR included 3ul of LNA™ SYBR® Green Master Mix 0.3ul of LNA™ miRNA specific primer and 0.3ul of Universal PCR primer Real-time quantitative PCR was performed with the Sequence Detection System 7900HT (Applied Biosystems) using the miRCURY LNA™ SYBR® Green Master Mix (Exiqon) following the manufacturer's instructions. The amplification conditions consisted of an initial step at 95 ºC for 10 min, followed by 50 cycles of 20 s at 95 ºC and 1 min at 60 ºC. Negative controls were included to all series of PCRs and all assays were performed in triplicates. All experiments were performed in triplicate and the mean of triplicates was used. Normalization was done with SNORA66 RNA and 5S rRNA. Relative expression was calculated using the comparative Ct method.

4.3. Microarray hybridization

4.3.1. mRNA expression profiling

Messenger RNA expression profiling was done with Agilent 44 K Whole Human genome Oligo Microarray (Agilent Technologies, Palo Alto, CA) containing 45,015 features representing 41,000 unique probes in a dual-color reference sample experimental design. cDNA preparations, hybridizations, washes and detection were done as recommended by the supplier. For whole genome transcriptional profiling, total RNA from samples and Universal Human Reference RNA (Stratagene, La Jolla, CA, USA) were used for amplification and labeling using the Low RNA Input Linear Amplification Kit (Agilent Technologies) following the Enhanced Oligonucleotide Microarray Labeling and Hybridization protocol established by Genomic Unit at the Spanish National Cancer Research Centre. Briefly, 500ng of total RNA was reversed transcribed to double-strand cDNA using a poly dT-T7 promoter primer. At first, template RNA and quality-control transcripts of known concentration and quality were denatured at 65ºC for 10 min and incubated for 2 hours at 40ºC with 5x First strand Buffer, 0.1 M DTT, 10 mM dNTP, MMLV RT, and RNase-out. The MMLV RT enzyme was inactivated at 65ºC for 15 min. cDNA products were then used as templates for in vitro transcription to generate fluorescent cDNA. cDNA products were mixed with a transcription master mix in the presence of T7 RNA polymerase and Cyanine 5 (Cy5) labeled or Cyanine 3 (Cy3) labeled-CTP and incubated at 40ºC for 2 hours. Labeled

cRNAs were purified using QIAGEN's RNeasy mini spin columns and eluted in 30ul of nuclease-free water. For each hybridization equal amounts of Cy3 labeled cRNA (reference) and of Cy5 labeled cRNA (samples) were mixed, fragmented, and hybridized onto an Agilent 4×44 K Whole Human genome Oligo Microarray. Specifically, 1ug of Cy3 labeled cRNA (reference) and 1ug of Cy5 labeled cRNA (carriers or controls) were mixed along with 10x Blocking Agent and 25x Fragmentation buffer to a final volume of 45ul. After 30 minute incubation at 60C the RNA fragmentation step was stopped by adding 2XGE Hybridization buffer HI-RPM.

4.3.2. miRNA expression profiling

Microarray expression profiling of microRNAs was performed using miRCURY LNA™ microRNA Array v.11.0 – hsa, mmu & rno (Exiqon A/S, Vedbaek, Denmark), in a single-color experimental design. The miRCURY LNA™ microRNA Array v.11.0 – hsa, mmu & rno contains capture probes for all microRNAs in human, mouse, rat and their related viruses as annotated in miRBase Release v.11.0 including 1940 capture probes, in 4 replicates, representing 831 human miRNAs annotated in miRBasev.11 database and 434 hsa- miRPlus probes (Exiqon proprietary). Forty three control capture probes are included in the probe set (ten spike-in control probes to ensure optimal labeling and hybridization, seven negative control capture probes and twenty six capture probes complementary to small nuclear RNAs). Labeling and hybridization procedure was performed as recommended by manufacturer, using miRCURY LNA™ microRNA Power Labeling Kit (Exiqon). First, 300ng of total RNA was treated with Calf Intestinal Alkaline Phosphatase (CIP) to remove the 5'-phosphates from the microRNA termini prior to labeling with Hy3 green fluorescent dye. Second, a Hy3 fluorescent label is attached enzymatically to the 3'-end of the microRNAs in the total RNA sample. This is followed by an enzyme inactivation step after which the sample is ready for hybridization. Labeling reaction was performed using 2ul of CIP treated total RNA, 1.5ul of Hy3 fluorescent dye, 2ul DMSO and 2ul of labeling enzyme, reaction was incubated at 16ºC for 1h and heat inactivated by incubation at 65ºC for 15 minutes and left at 4ºC until hybridization step. Labeled samples were subsequently loaded onto a miRNA microarray slide and hybridized over 16h at 56ºC. A set of 10 synthetic spike-in RNAs were added to total RNA sample prior to labeling and later used for quality control for RNA labeling reaction and inter-array reproducibility. Washing of the slides was performed according to manufacturer´s instruction. Washed slides were dried by centrifuging at 1200 rpm for 5 minutes.

4.3.3. Array scanning and microarray data extraction

Processed slides were scanned with Agilent Array scanner (Agilent Technologies), with the laser set to 635nm, at Power 80 and PMT 70 setting, and a scan resolution of 10μm. To avoid ozone bleaching, microarrays were scanned in an ozone-free environment (less than 2 ppb ozone). Fluorescence intensities on scanned images were quantified using Agilent Feature Extraction software (version 9.5.3) (Agilent Technologies). For miRNA data extraction we have used the modified Exiqon protocol, while for mRNA data extraction the GE2-v5_95_Feb07 protocol was used. Reproducibility and reliability of each single microarray was assessed using Quality Control report data.

5. MICROARRAY DATA ANALYSIS

5.1. Background subtraction and log transformation

Background subtraction is the first step in adjusting the raw data towards comparability. It has consistently been found to correct log ratios preventing underestimation of the observed changes in signal intensities. Average values of the replicate spots were background subtracted using Normexps background correction method that is a convolution model assuming that background signals are normally distributed while the sample signals are exponentially distributed. Processed intensity data from miRNA array were than log2 transformed and subjected to further analysis. For mRNA expression data, expression ratios were calculated (Cy5 processed signal was divided by Cy3 processed signal) and log2 transformed.

5.2. Deposition of microarray raw data in public databases

Microarray datasets used in the study have been deposited at Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/info/linking.html) under GEO accession number: GSE30822, and GSE32922.

5.3. Microarray data normalization

When running experiments that involve multiple high density oligonucleotide arrays, it is important to remove sources of variation between arrays of non-biological origin, such as dye bias and differences in labeling, hybridization and scanning, which can occur when performing an array experiment. Normalization is a process for reducing this variation and to make data of different sources comparable. Normalization is performed on data from individual arrays (intra-slide normalization) and on data from a set of arrays (inter-slide normalization).

Expression data from mRNA microarray were normalized using Lowess and quantiles methods for intra-array and inter-array normalization, respectively. Lowess (Locally Weighted Scatterplot Smoothing) is the use of a locally established regression to smooth the M/A (log ratio/log mean-intensity) scatterplot toward a linear distribution to eliminate the dye- and label-specific variances. Raw data obtained from miRNA microarray experiment were quantile normalized for inter-array variability. The Lowess algorithm works under the assumption that the majority of the signals between samples do not differ and it enforces equal overall means on all signal intensities. Quantile normalization is based on the assumption that two sets of closely related data should sort themselves in a linear fashion forming a diagonal when plotted against each other. The goal of the quantile method is to make the distribution of probe intensities for each array in a set of arrays the same and it enforces an equal intensity distribution on the data.

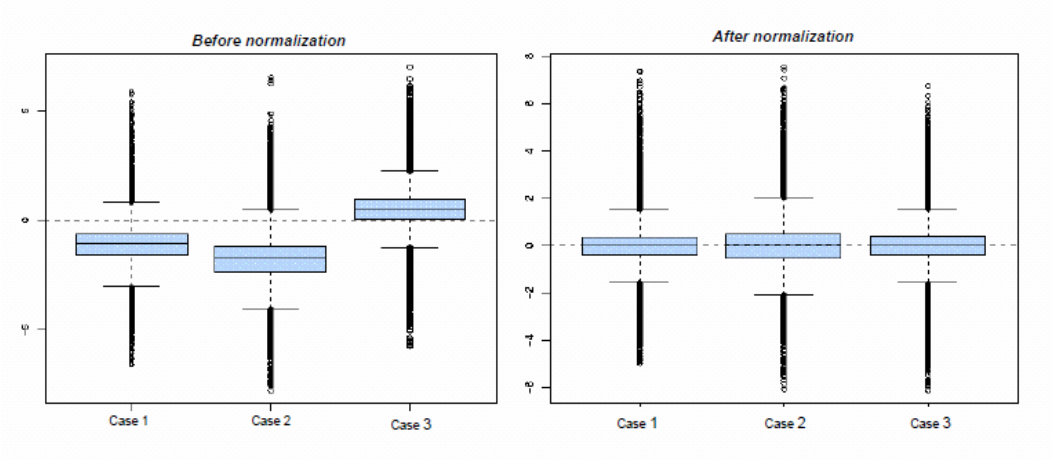


Figure 8. Expression data point distribution prior to and post normalization

5.4. Microarray data pre-processing

Gene patterns containing missing values were discarded. Additionally a filter procedure eliminated genes with uniformly low expression or with low expression variation across the experiments, retaining 20 374 genes and transcripts. miRNA data was preprocessed to eliminate miRNAs with uniformly low expression or with low expression variation (SD<0.3) across the experiments, retaining 466 miRNA genes (306 hsa-miR + 160 hsa-miRPlus).

6. BIOINFORMATIC AND STATISTICAL ANALYSIS

6.1. Class discovery analysis

Average linkage hierarchical cluster analysis using Pearson correlation with uncentered metrics was performed using Gene Cluster (Eisen et al., 1998) and data were visualized by Treeview v.1.6 (http://rana.stanford.edu/software; Stanford University, USA). The level of expression of each miRNA in each sample, relative to the median level of expression of that gene across all the samples was represented using a red-black-green color scale. Green corresponds to expression value below median, black equal to median, and red above the median.

Consensus Clustering module available in the Gene Pattern suite (Reich et al., 2006) was used for class discovery and clustering validation. This method facilitates the discovery of biologically meaningful clusters by assessing the stability of the discovered clusters by means of resampling techniques. The consensus clustering analyses were run with a KNN means algorithm was applied, with 2, 3, 4, and 5 centroids using 500 resampling iterations. The consensus among the multiple runs is assessed and summarized in a consensus matrix and ΔG plot. This matrix is used as a visualization tool to estimate the composition and number of the clusters, and the ΔG plot indicates the change in free energy with every additional group added.

6.2. Differential expression analysis

Differential expression analysis was performed with linear models (limma) implemented in the POMELO II tool, available in Asterias web server (http://asterias.bioinfo.cnio.es). The estimated significance level (p value) was corrected for multiple hypotheses testing using Benjamini & Hochberg False Discovery Rate (FDR) adjustment. Differential expression analysis was performed with linear models (limma) t-test implemented in the POMELO II tool, available in Asterias package (http://asterias.bioinfo.cnio.es) (Morrissey and Diaz-Uriarte, 2009). The estimated significance level (unadjusted p-values) were corrected for multiple hypotheses testing using Benjamini and Hochberg False Discovery Rate (FDR) adjustment (Benjamini et al., 2001). Those miRNAs with FDR <0.05 were selected as significantly differentially expressed, while for mRNAs, FDR<0.01 was set as threshold.

6.3. Databases and miRNA repositories

miRBase (http://microrna.sanger.ac.uk) is a central repository for all reported and experimentally detected miRNA genes(Griffiths-Jones, 2004; Griffiths-Jones et al., 2008). It was initiated at the Welcome Trust Sanger Institute and is currently hosted and maintained at the University of Manchester.

It comprises three services: miRBase:Sequences provides information about over 9000 miRNAs from 103 species, including the sequences and genomic locations of the miRNAs; miRBase:Targets provides miRNA target predictions based on the miRanda algorithm; and miRBase:Registry is a service that assigns names to novel miRNA genes according to a defined miRNA nomenclature.

Several publically available databases contain information on experimentally validated miRNA target genes obtained from literature data mining. The list of confirmed miRNA target genes was compiled from: Tarbase v.5c (DIANA lab, http://diana.cslab.ece.ntua.gr/tarbase/), miRecords (http://mirecords.biolead.org/) and miTarBase (http://mirtarbase.mbc.nctu.edu.tw/index.html).

6.4. miRNA target prediction

A number of computational prediction programs have been developed to identify putative miRNA targets based on sequence complementarity between the miRNA and its potential mRNA target 3' untranslated region (3'UTR) (Bartel, 2009). Near perfect complementarity in the seed region at the 5' end 2-8 nucleotide position of the miRNA seems to be the most important factor for miRNA target prediction (**Figure 9** left). Furthermore pairing to the 3' region of the miRNA can also compensate for a mismatch in the seed region. These so called "3'-compensatory sites" are centered on miRNA nucleotides 13-17 (**Figure 9** right). In an attempt to increase target prediction specificity some prediction algorithms are relying on target site evolutionary conservation and thermodynamic stability of the RNA-RNA duplex. To determine potential mRNA targets for specific miRNAs we have used several publically available target prediction algorithms, namely, miRanda (microRNA.org), TargetScanS (www.targetscan.org), DIANA microT (http://diana.cslab.ece.ntua.gr/microT/) and PicTar (http://pictar.mdc-berlin.de/).

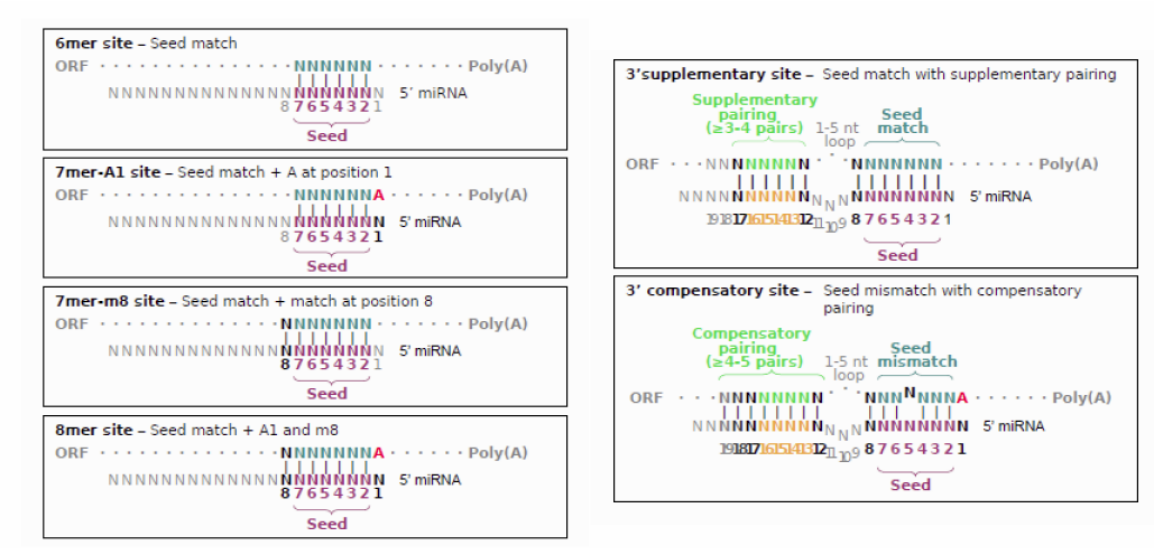


Figure 9. Types of miRNA target sites. Adapted from Bartel DP, Cell 2009.

6.5. Integration of miRNA and mRNA gene expression signatures

For each differentially expressed miRNA, a contingency table relating the miRNA and its predicted gene targets was produced using miRBase Targets Release v. 5.0 (http://www.mirbase.org/; Faculty of Life Sciences, University of Manchester), taking into account whether these targets were included in a consistent gene expression signature (down-regulated targets for upregulated miRNAs and *vice versa*). Fisher's exact test was used for significance analysis. Those miRNAs whose Fisher's exact test result indicated a $p < 0.05$ were selected for further analysis on the basis of their non-random association with the gene expression signature of interest. To identify statistically significant associations between differentially expressed miRNAs (both t-test and Fisher's exact test $p<0.05$) and enriched pathways (FDR< 0.05), a ranked list was built. Down-regulated miRNAs were tested for their association with upregulated genes, whereas upregulated miRNAs were tested for their association with down-regulated genes. The ranked target list of the differentially expressed genes was subjected to pathway enrichment analyses using Ingenuity Pathway Analysis 7.6 software (Ingenuity Systems, Redwood City, CA). Interaction networks were built and depicted using Cytoscape bioinformatics software (Shannon et al., 2003) (http://www.cytoscape.org).

6.6. Functional annotation and gene set enrichment analysis

Several different bioinformatic tools have been used for biological interpretation and the analysis of pathways associated to defined lists of genes. These included DIANA miRPath, Ingenuity Pathway Analysis® and DAVID.

Ingenuity Pathway Analysis® (http://www.ingenuity.com/products/pathways_analysis.html) was used to gain insight into global molecular networks and canonical pathways specific to significantly associated miRNA-mRNA gene pairs differentially expressed between BRCA1 proficient and deficient cells. The ranking of genes was performed with t-test, with an absolute mode for gene list sorting. Gene sets are defined based on prior biological knowledge, e.g., published information about biochemical pathways (KEGG, Biocarta, Reactome) or co-expression in previous experiments, and are manually curatted and stored in the Ingenuity Knowledge Database®. False discovery rate (FDR, q value) is computed in by the IPA program. Those pathways showing FDR <0.15, a well-established cut-off for the identification of biologically relevant gene sets (Subramanian et al., 2005), were considered significantly enriched between classes under comparison. Pathways that have less than 15 gene members or over 500 were excluded from the analysis.

DIANA miRPath pathway enrichment analysis (Papadopoulos et al., 2009)(http://diana.cslab.ece.ntua.gr/) was used to gain insight into global molecular networks and canonical pathways related to differentially expressed miRNAs between normal and tumor samples.

DIANA miRPath is a web-based computational tool developed to identify molecular pathways potentially altered by the expression of single or multiple microRNAs. The software performs an enrichment analysis of multiple microRNA target genes comparing each set of microRNA targets to all known KEGG pathways. Those pathways showing p-value <0.05, were considered significantly enriched between classes under comparison.

The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 bioinformatic algorithm (http://david.abcc.ncifcrf.gov/) was used for the functional annotation analysis of Gene Ontology (GO) terms associated with a given gene list of confirmed mRNA targets within each miRNA cluster specific for different BRCAX tumor subtypes (Huang da et al., 2009). GO functional categories with p-values under 0.05 after correction for multiple hypotheses testing by Benjamini and Hochberg were considered as statistically significant.

RESULTS

In continuation, results obtained during the course of this thesis, from January 2009 until June 2012, shall be presented. In total three different studies have been performed, implementing various techniques including miRNA and mRNA expression profiling, IHC, and cell-based functional studies in various series of breast tissue samples, as described in Materials and Methods. In summary:

The first study is focused on the integration analysis of miRNA and mRNA expression profiles, and identification of BRCA1-modulated pathways and miRNA target genes in two isogenic cell lines, proficient and deficient for BRCA1 expression. (Tanic et al, *Breast Cancer Res Treat,* 2011)

The second study is centered on the analysis of miRNA expression profile in a series of 36 fresh-frozen breast tissues (22 breast tumors and 14 normal breast tissues), describing miRNAs deregulated in tumors *versus* normal breast tissues, identifying deregulated pathways and novel miRNA target genes. (Tanic et al, *PloS One,* 2012)

The third and the final study is based on the miRNA expression profiling in a series of 80 FFPE breast tissues, focusing on the analysis of miRNAs differentiating between different genetic subgroups, BRCA1 and BRCA2 mutation carriers, BRCAX and sporadic breast tumors, and the analysis of BRCAX tumor heterogeneity, resulting in a miRNA-based molecular sub-classification of BRCAX tumors.

RESULTS PART I

1. INTEGRATION OF BRCA1-MODULATED MIRNA AND MRNA EXPRESSION PROFILES

Germline *BRCA1* mutations are a well known factor of susceptibility to breast and ovarian cancers (Bertwistle and Ashworth, 1998; Rahman and Stratton, 1998). Somatic inactivation of *BRCA1* is common in sporadic breast cancer with ~40% of sporadic breast tumors showing decreased levels of BRCA1 mRNA/protein expression (Esteller M, 2000;Turner, 2007; Wei M, 2008), with approximately 20% of sporadic breast tumors showing complete loss of BRCA1 protein expression (Taylor J, 1998), emphasizing the importance of BRCA1 deregulation in breast pathogenesis. Incredible versatility of BRCA1 functions has been demonstrated so far, including DNA damage repair/recombination, cell cycle control (Deng, 2006), chromatin remodeling and ubiquitylation (Huen et al., 2010) and regulation of both transcriptional activation and repression (Cable et al., 2003; Chapman and Verma, 1996).

The first objective of this thesis project was to identify miRNAs and miRNA-mRNA interaction networks dependant on BRCA1 expression status. By integration analysis of genome-wide miRNA and gene expression data determined by microarray technology, we aimed to reveal underlying biological processes and pathways, and to identify new miRNA target genes potentially implicated in BRCA1-associated tumorigenesis.

1.1. Study material and model system of BRCA1 reconstitution

The model system used in this study consisted of paired isogenic cell lines deficient and proficient for BRCA1 expression, HCC1937 cell line derived from hereditary *BRCA1* mutated breast tumor and HCC1937/BRCA1^{wt} stably expressing full length wild-type *BRCA1* mRNA (Huang et al., 2009; Huen et al., 2007), that were kindly provided by Dr. J. Chen (Yale University School of Medicine Department of Therapeutic Radiology, New Haven, USA) (**Figure 10**). In addition 13 formalin fixed paraffin embedded (FFPE) tumor tissues from hereditary breast cancer cases harboring *BRCA1* mutations, as well as 4 normal breast tissues from mammary reduction surgery, were selected to check the expression of interesting miRNAs.

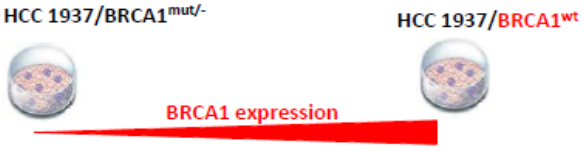


Figure 10. Cell model system used for the study - cell line HCC 1937/BRCA1$^{mut/-}$ and its isogenic counterpart with reconstituted wild-type BRCA1 protein expression HCC 1937/BRCA1^{wt}.

1.2. Significantly modulated mRNAs and miRNAs in response to BRCA1 reconstitution

To determine the effect of BRCA1 reconstitution on miRNA and mRNA profiles, whole genome transcriptional profiling and global miRNA expression profiling was performed in the BRCA1-deficient HCC1937 breast cancer cell line and its isogenic counterpart HCC1937/BRCA1^{wt}, stably expressing wild-type BRCA1. A total of 8845 genes demonstrated statistically significant (FDR <0.01) differential expression, representing a 26% shift in global gene expression, with 4710 transcripts downregulated and 4135 transcripts upregulated in BRCA1-proficient cells relative to BRCA1-deficient HCC1937 breast cancer cells (**Figure 12A**). The full list of differentially expressed genes is shown in **Supplementary Table 3**.

In contrast, miRNA expression profiles exhibited very high similarity in the two isogenic cell lines, with a Pearson correlation coefficient of R^2=0.93 (Figure 1c). Differential expression analysis showed that only 9 miRNAs (miR-99a, miR-99b, miR-138, miR-146a, miR146b, miR-205, miR-302b*, miR-923 and miR-1290) were significantly differentially expressed between the cell lines with FDR <0.05, representing 1% change in global miRNA expression upon BRCA1 reconstitution (**Figure 12B**). Most of these miRNAs were upregulated in HCC1937/BRCA1^{wt} compared to HCC1937, while only miR-138 was down-regulated in HCC1937/BRCA1^{wt}. Quantitative RT-PCR analysis performed on selected miRNAs confirmed the pattern of differential expression shown by microarray analysis (**Figure 11)**.

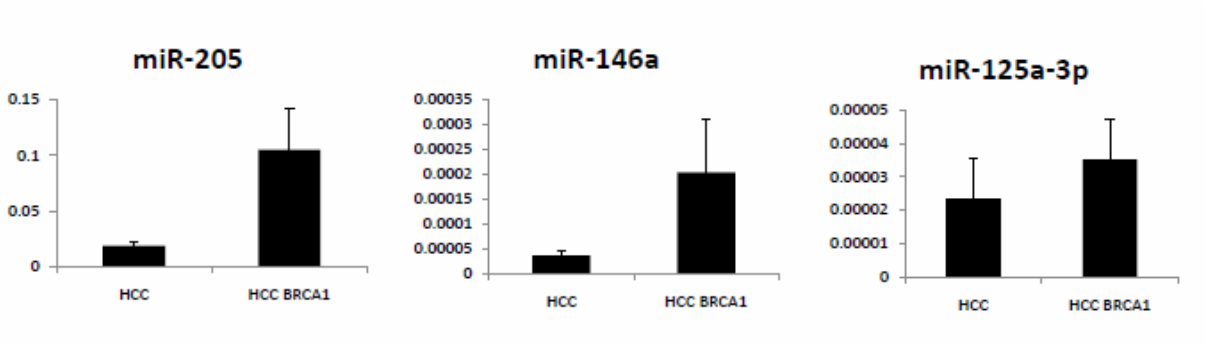


Figure 11. Quantitative RT-PCR validation of microarray results demonstrating consistently reduced expression of miR-205, miR-146a and miR-125a-3p in HCC 1937 cells in comparison to HCC1937/BRCA1^{wt}.

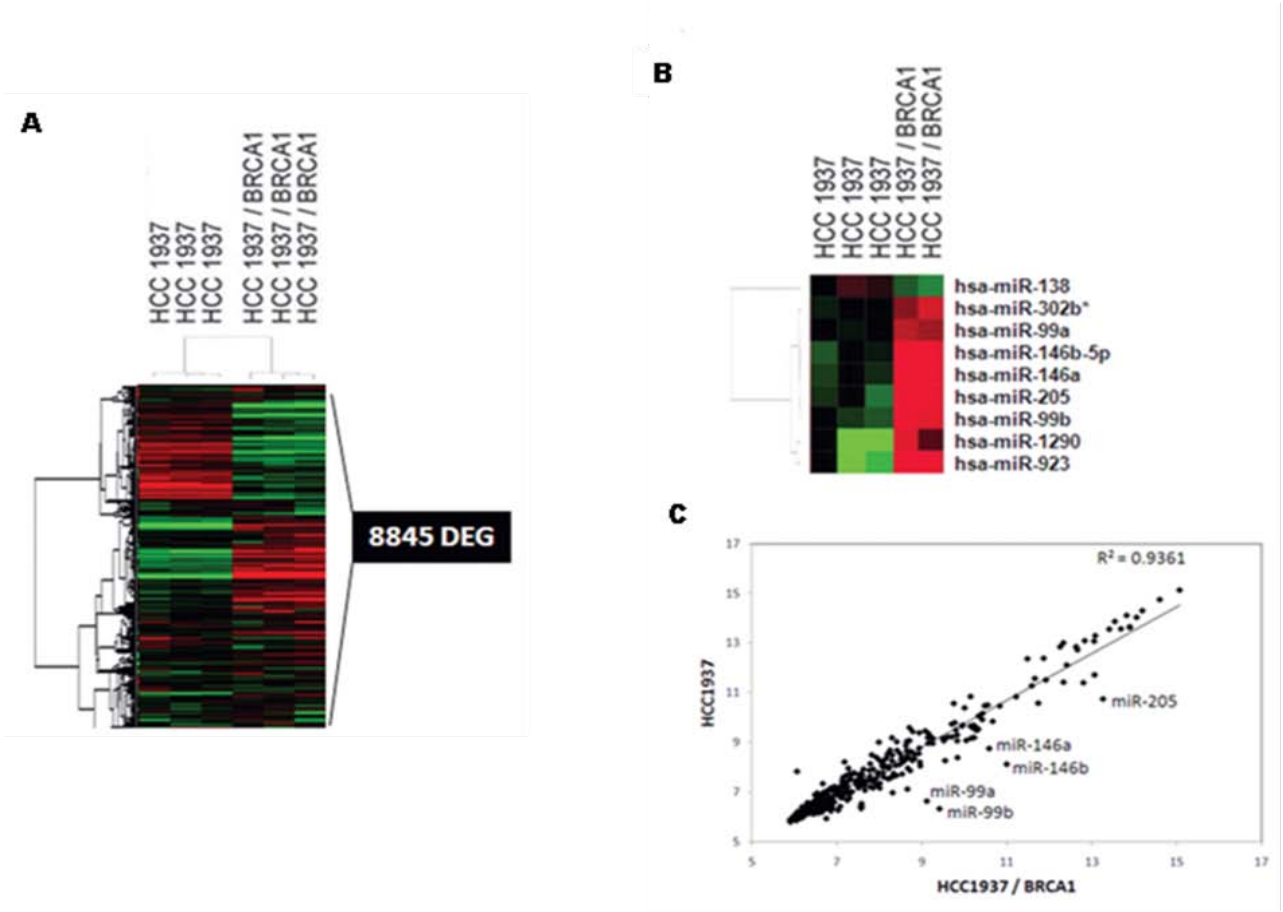


Figure 12. mRNA and miRNA transcriptional changes upon BRCA1 reconstitution. **A)** mRNA expression heatmap of differentially expressed genes (DEG) with FDR<0.01 between HCC1937 and HCC 1937/BRCA1^{wt} cells. Upregulated genes in HCC1937/BRCA1^{wt} are shown in red, downregulated genes in green. **B)** Differentially expressed miRNAs (FDR<0.05) between HCC1937 and HCC 1937/BRCA1^{wt} cells. Upregulated miRNAs in HCC1937/BRCA1^{wt} are shown in red, downregulated in green. **C)** Correlation plot between miRNA expression in HCC1937 and HCC 1937/BRCA1^{wt} cells. Linear regression analysis of global miRNA expression in the two isogenic cell lines; squared correlation coefficient of R^2=0.93 indicates high similarity of the two datasets.

1.3. miRNA and mRNA integration analysis reveal significant miRNA-mRNA target pairs and pathways

Once the initial observation that miRNAs are differentially expressed between BRCA1-proficient and deficient cells; an integrated analysis of the miRNA and mRNA expression profiles was carried out to identify putative miRNA targets. The integrated analysis of miRNA and mRNA expression levels has been based on the assumption that increased or decreased expression of a specific miRNA results in at least partially decreased or increased expression of corresponding target mRNAs (Lim et al., 2005). To identify statistically significant associations between differentially expressed miRNAs (DEmiRs, FDR<0.05) and differentially expressed genes (DEGs, FDR<0.01), we tested whether predicted miRNA-mRNA targeting pairs were non-randomly associated by Fisher's exact test (**Figure 13)**. Gene target predictions for human miRNAs were obtained using miRBase Targets Release v5.0 (http://www.miRBase.org, Faculty of Life Sciences, University of Manchester). Results of Fisher's exact test indicated that miR-138, miR-146a, miR-146b and miR-99b were significantly associated with differentially expressed mRNA targets within the gene expression signature. Significant miRNAs upregulated in HCC1937/BRCA1^{wt} (miR-146a, miR-146b and miR-99b) were collectively targeting 160 downregulated DEGs. Interestingly, five genes *SURF1, TRAF2, FBXW2, HIST1H4I* and *MARK1* were identified as common targets for all 3 significant upregulated miRNAs. On the other hand miR-138 was significantly associated to 75 upregulated DEG.

To explore the biological significance for observed changes, significant miRNA-mRNA target pairs were evaluated for representation of relevant functional biological processes and pathways using Ingenuity Pathway Analysis. The pathway analysis over the 160 significant downregulated DEG revealed statistically significant enrichment for 15 pathways related to proliferation signaling and inflammation. Among the most interesting were p38/MAPK, MAPK/JNK and NF-κB signaling pathways. Interestingly, *TRAF2* (TNF receptor associated-factor 2) gene, a well established mediator of both NF-κB and MAPK pathway activation (Carpentier et al., 1998), was predicted to be a target of the 3 significant miRNAs (miR-146a, miR-146b and miR-99b) along with another two out of the 9 differentially expressed miRNAs, miR-99a and miR-205. On the other hand, there were only two pathways (Clathrin-mediated Endocytosis Signaling and IL-10 Signaling) that were significantly enriched within 75 significant upregulated DEG, targets of miR-138. Full list of pathways is represented in **Supplementary Table 4**.

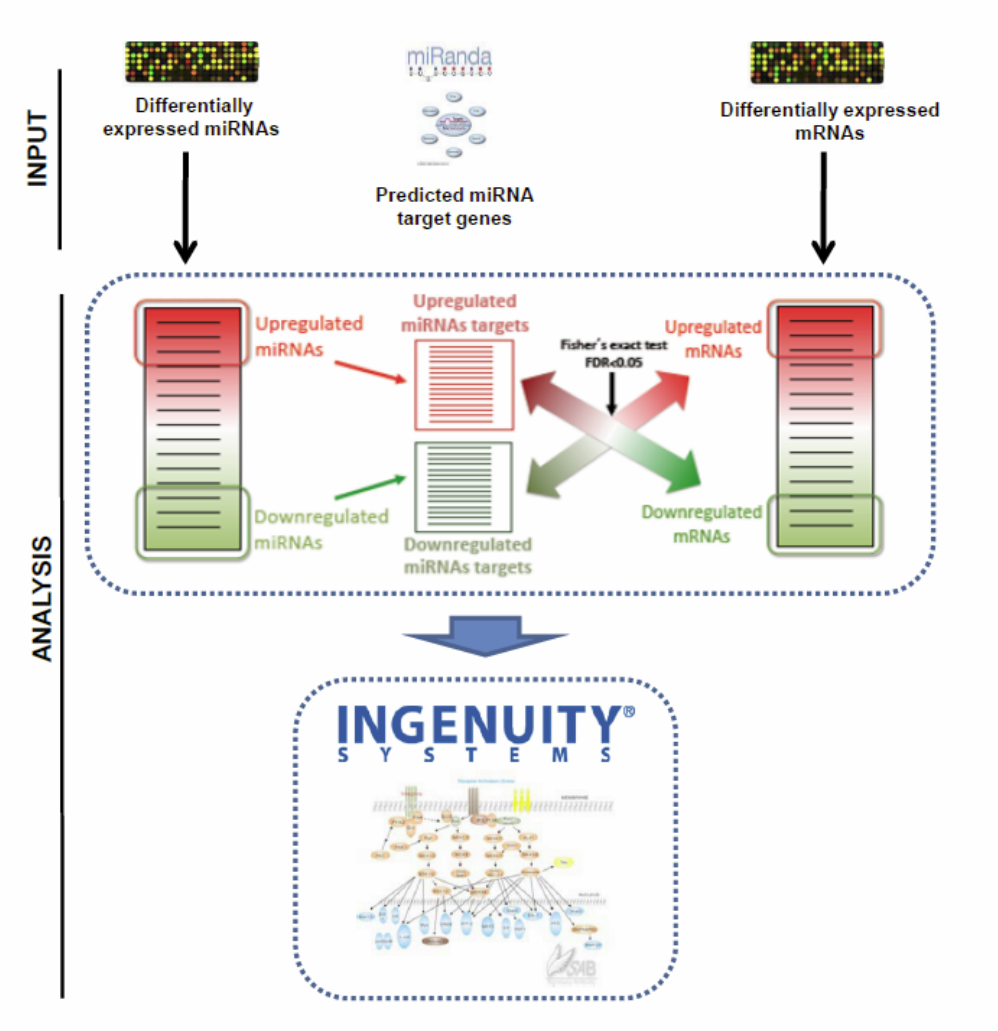


Figure 13. Outline of miRNA and mRNA expression integration analysis following BRCA1 gene re-expression. Significantly differentially expressed miRNA (FDR<0.05) and mRNAs (FDR<0.01) upon BRCA1 expression, were investigated the existence of inverse correlation between upregulated expression of a microRNA and its predicted mRNA target genes. Gene target predictions for human miRNAs were obtained using miRBase Targets Release v5.0 implementing miRanda target prediction algorithm. Those miRNAs found to be significantly associated on the basis of the Fisher's exact test (FDR<0.05) with the gene expression signature of interest were selected. Ingenuity Pathway Analysis (IPA) was used to find enriched pathways for the mRNA targets significantly associated to differentially expressed miRNAs. Functional validation of the significantly enriched pathways (FDR<0.05) was performed with Luciferase reporter assay.

An interaction network (**Figure 14**) was built to represent connections of significant upregulated DEmiRs, their DE-mRNA targets and the associated pathways. This approach allowed us to infer experimental microarray gene expression data to predict the effects of miRNA expression on the global behavior of biological pathways.

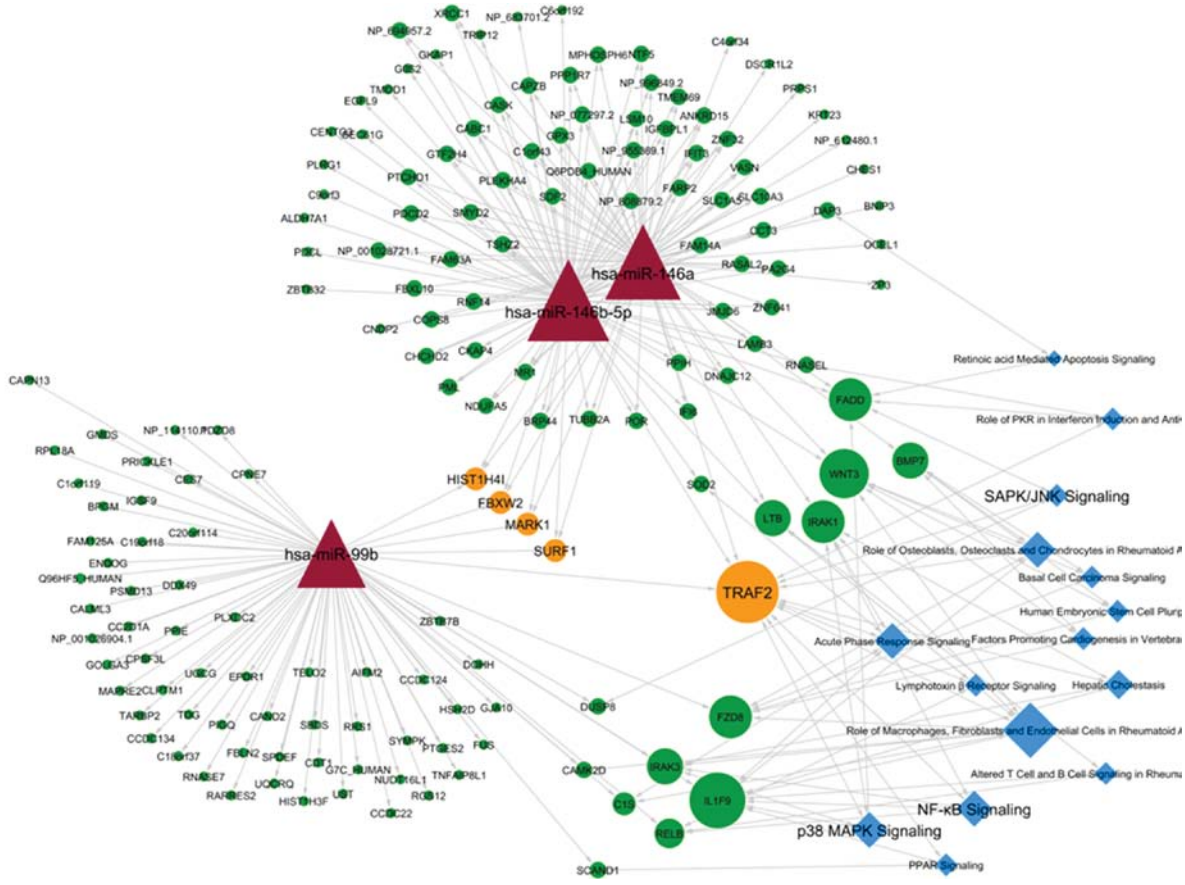


Figure 14. Interaction network showing upregulated miRNAs in HCC 1937/BRCA1^{wt} cells with connections to downregulated genes and pathways**.** miRNAs are indicated by red triangles, miRNA target genes are depicted as green (1-2 connections of coding genes to miRNAs) and yellow circles (3 connections), whereas pathways are represented by blue diamonds. The size is proportional to their degree of connectivity. All the connections represent statistically significant relationships with FDR<0.05 set as threshold.

1.4. Changes in pathway activation between BRCA1-profficient and deficient HCC1937 cells

We quantitatively assessed signal transduction pathway activation by measuring the activities of downstream transcription factors *in vitro* in the isogenic cell lines deficient and proficient for BRCA1. Transcription factor (TF) responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal (m) CMV promoter and tandem repeats of the TF transcriptional response element (TRE). Reporter transcription factors used for assessment of NFκB, MAPK/ERK, MAPK/JNK pathway signaling, were NFκB, Elk-1/SRF and AP-1, respectively; Interestingly, the results revealed that MAPK/JNK pathway were significantly down-regulated in HCC1937/BRCA1^{wt} compared to the BRCA1-null HCC1937 cell line (**Figure 15**). In addition, NF-κB and MAPK/ERK showed also reduced activity when *BRCA1* was expressed in HCC1937 cells, although this was not statistically significant. Reduction in the *in vitro* activity of these pathways supports the findings obtained by Ingenuity Pathway analysis.

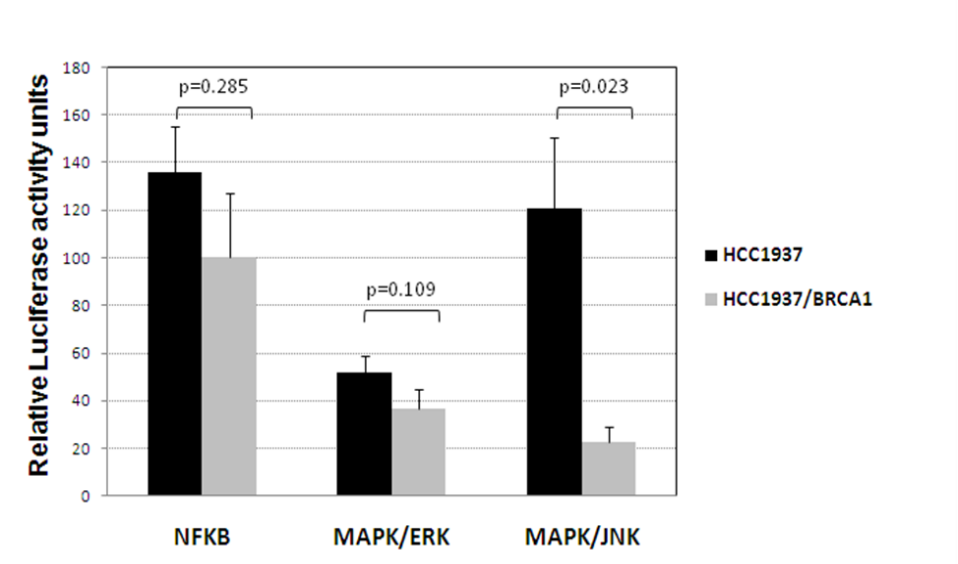


Figure 15. Luciferase reported assay for activation of signaling pathways. MAPK/ERK, MAPK/JNK and NFκB signaling pathways show decreased activation upon BRCA1 reconstitution. Pathway activation in the BRCA1-deficient and proficient isogenic HCC1937 cell lines was assessed by measuring the activities of downstream transcription factors.

Consistent with this finding, these cell lines had markedly different proliferation rates, with *BRCA1*-deficient cells proliferating considerably faster than their isogenic *BRCA1*-proficient counterparts (**Figure 16**), suggesting that the increased activity of the NF-κB and/or MAPK pathways in HCC1937 cells might play an important role in cell survival and proliferation in context of *BRCA1*.

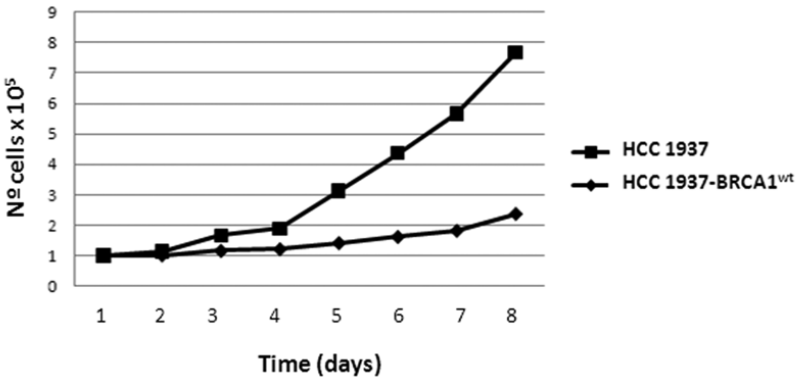


Figure 16. Growth curves of HCC1937 and HCC1937 BRCA1-expressing cells. BRCA1 reconstitution impacts proliferation of HCC1937 cells. $BRCA1^{wt}$-expressing HCC1937 cells showed a lower increase in the number of growing cells comparing to the HCC1937 BRCA1-null cells.

1.5. TRAF2 is regulated by miR-146, miR-99 and miR-205

The computational analyses revealed *TRAF2* gene as a common target for 5 out of 9 differentially expressed miRNAs, miR-146a, miR146b, miR-99a, miR-99b and miR-205, with several predicted binding sites in its 3'UTR (**Figure 18A**). In addition, we observed that *TRAF2* had higher endogenous expression, at both protein (**Figure 18C**) and mRNA level (**Figure 17**), in HCC1937, which expresses lower levels of miR-146a/b, miR-99a/b and miR-205. To validate *TRAF2* as a *bona fide* target of these miRNAs we performed luciferase reporter assays to check binding of these miRNAs to the 3'UTR of *TRAF2* gene. Cells transfected with miR-146 showed 50% reduction in normalized luciferase signal compared to scramble control, while miR-99 and miR-205 transfected cells exhibited approximately 30% decrease in luciferase activity (**Figure 18B**). Combination of all three miRNAs did not affect any further the expression of *TRAF2*.

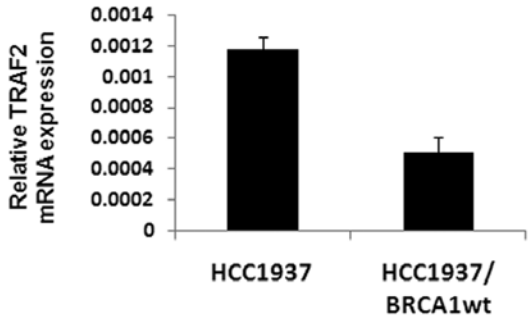


Figure 17. mRNA levels of TRAF2 in HCC1937 and HCC1937/BRCA1^{wt} cells. Endogenous TRAF2 mRNA levels are higher in BRCA1-null HCC1937 than HCC1937/BRCA1^{wt}. Bars represent mean expression values and error bars represent standard deviation from three different experiments.

To study the effect of miRNA on the *TRAF2* mRNA and protein level changes, we have transfected HCC1937 cells with pre-miRNA oligonucleotides for miR-146a, miR-99b and miR-205. Although mRNA levels of *TRAF2* did not show a significant changes after miRNA re-expression (**Figure 8C**), western blot analyses demonstrated a reduction in *TRAF2* protein levels in cells transfected with pre-miR-146a, pre-miR-205, and pre-miR-99b, compared to scramble control and mock transfection (**Figure 8C**). Collectively, luciferase reporter assay and western blot results showed that miR-146a, miR-99b and miR-205 act as negative regulators of *TRAF2* expression.

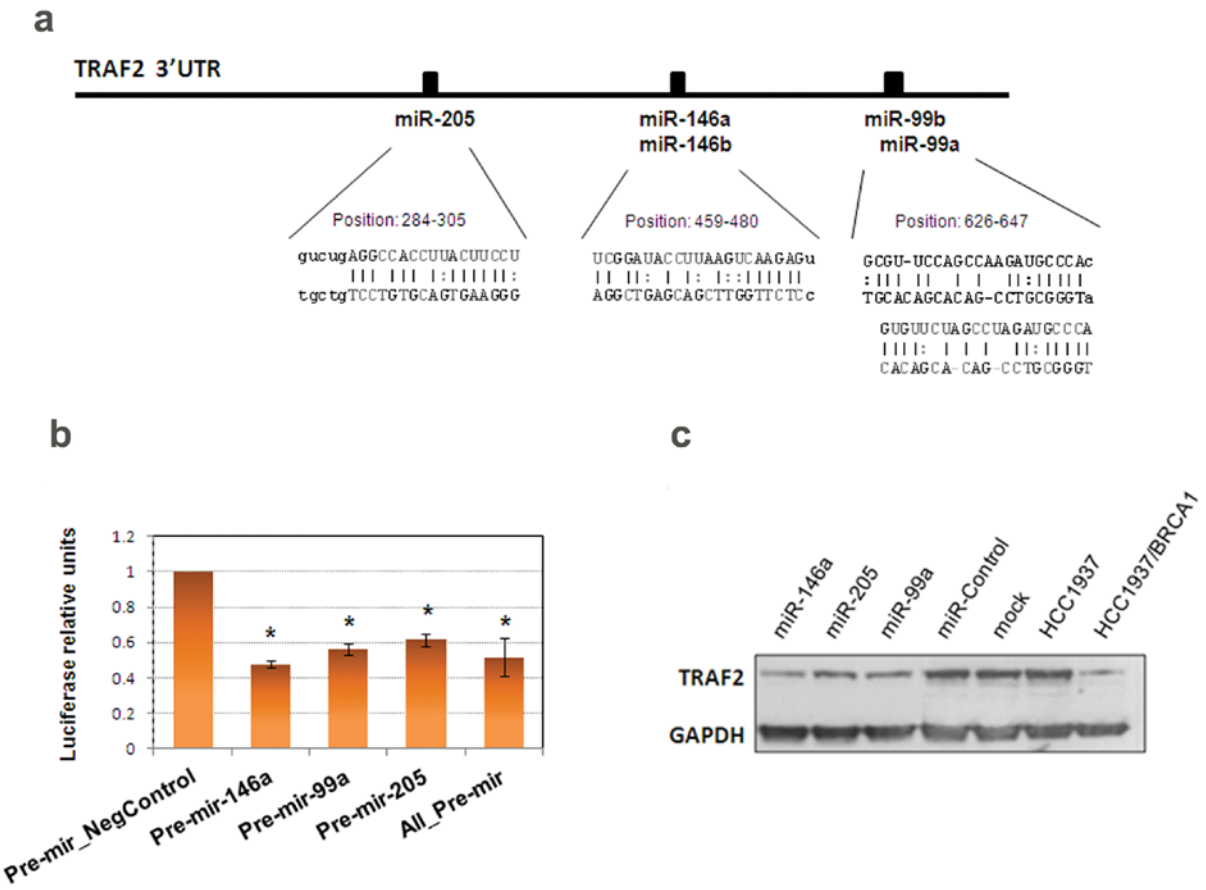


Figure 18. Negative regulation of TRAF2 expression by miR-146a, miR99a and miR-205. **A)** Schematic representation of miRNA binding sites within the TRAF2 3'UTR region. Complementary binding sites for miR-146a, miR-146b, miR-99a, miR-99b and miR-205 are based on miRanda target prediction software. **B)** Luciferase activity of a reporter construct carrying the TRAF2 3'-UTR downstream of the luciferase gene. The construct was co-transfected with each of the indicated miRNA precursors and pool combination of all 3 pre-miRNAs into HCC1937 cells. The pool contains an equimolar mixture of all indicated miRNAs in which the sum of all these miRNAs equals 50nM as in the scramble vectors or the previous assays. All data are normalized versus the luciferase levels generated by scramble sequences. (* , $p<0.05$) **C)** TRAF2 protein levels after miRNA expression. The effect of miR-146a, miR-99a and miR-205 on TRAF2 protein levels was assessed by western blot. HCC1937 cells were transfected with 50nM miR-146a, miR-99a, miR-205 or scramble control, or mock transfected. TRAF2 levels in non-transfected HCC1937 and HCC1397/BRCA1^{wt} are also represented. GAPDH was used as a loading control.

1.6. NF-κB pathway activity is modulated by miR-146, miR-99 and miR-205.

In order to determine whether miR-146a, miR-99b and miR-205 could have a direct effect on the NF-κB, MAPK/ERK and MAPK/JNK pathway activation, we re-expressed these miRNAs individually in HCC1937 cells and assayed for signaling pathway activation using the Multi-Pathway Reporter Assay. Our results demonstrated that all three miRNAs significantly reduced NF-κB pathway activity compared to negative control (**Figure 19**). Similarly, MAPK/ERK pathway exhibited significant reduction in activity only upon transfection with miR-146a. On the other hand, MAPK/JNK pathway showed no differences in activity levels compared to negative control, indicating that expression of just one miRNA is not sufficient to modulate activity of this pathway.

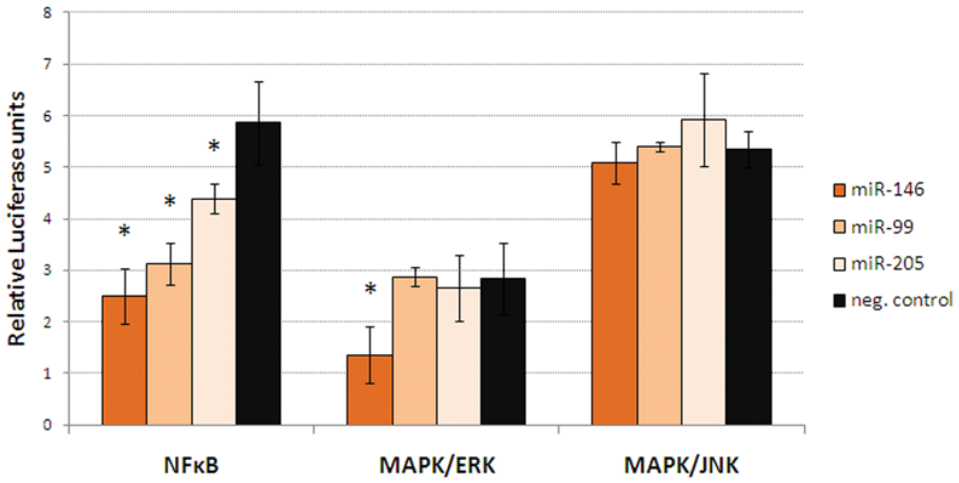


Figure 19. miRNA expression effects on pathway activation. Box plots showing the effect of miR-146a/b, miR-199a/b and miR-205 on NFκB, MAPK/ERK, MAPK/JNK pathway activation was assessed by measuring the activities of downstream transcription factors in the BRCA1-deficient HCC1937 cell line transfected with 200pm of each of these miRNAs. (*$p<0.05$)

1.7. Down-regulation of miR-99a, miR146b and miR-205 in primary BRCA1-mutated breast tumors.

To further explore the significance of miRNAs found to be associated with *BRCA1* re-expression in HCC1937, their level of expression was analyzed in a group of 13 *BRCA1* mutated primary tumors comparing to expression in normal breast tissue. Significant down-regulation of three of these miRNAs, miR-99a, miR146b and miR-205 was confirmed in BRCA1 tumors (**Figure 10**), indicating that these BRCA1-dependent miRNAs could be important also for tumor development *in vivo*.

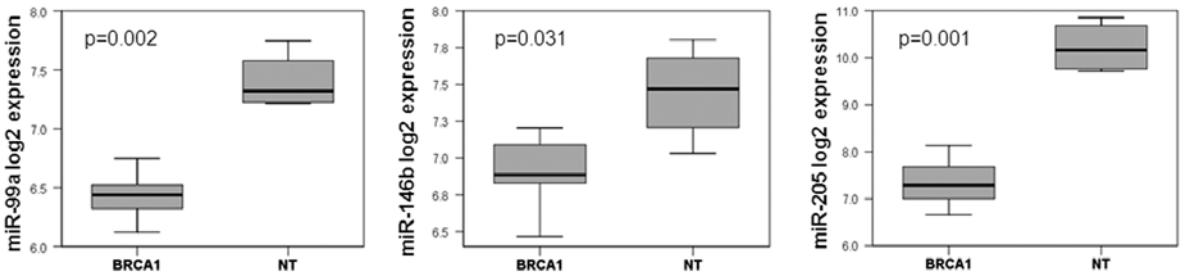


Figure 20. Box plots representing level of expression determined by microarray in a set of 13 BRCA1-mutated tumors comparing to normal breast tissue (NT) for three significant miRNAs, miR-99a, miR-146b, and miR-205 showed significant down regulation in tumors.

RESULTS PART II

2. DEREGULATED MIRNAS IN HEREDITARY BREAST CANCER

2.1. miRNA expression profiling in primary hereditary breast tumors and normal breast tissue.

miRNA deregulation in sporadic breast carcinomas has been well established, but to date, there are no reports describing miRNA deregulated in hereditary breast tumors. In order to establish the miRNA profile of hereditary breast tumors, we used LNA based microRNA microarrays. After initial preprocessing we obtained data from 1276 hsa-miRNAs (831 hsa-miR and 434 hsa-miRPlus). A filter procedure to eliminate genes with low expression variation across the experiments (SD<0.3) and with uniformly low expression, reduced the number of miRNAs to a total of 327 hsa-miRNAs (198 hsa-miR and 118 miRPlus). In an effort to detect significant differences in miRNA expression between normal breast tissue and hereditary breast tumors, we performed a differential expression analysis. We identified 19 miRNA significantly differentially expressed (FDR<0.05) between normal breast tissue and hereditary tumoral samples (**Table 3**). Almost all differentially expressed miRNAs were found to be down-regulated in tumor tissues, with the exception of miR-21 and miR-300 that were up-regulated in breast tumors compared to normal breast tissue. Among miRNAs that demonstrated more than two-fold changes in expression in hereditary breast tumors were miR-99a, miR-100, miR-195 and miR-143, while miR-205 and miR-125b showed almost 6-fold lower expression in hereditary tumors.

Table 3. miRNAs differentially expressed between normal breast and hereditary tumor tissue

miRNA	Chromosomal location	Median Normal Breast	Median Tumor Tissue	Fold change	Unadjusted p value	FDR- adjusted p value
hsa-miR-205	1q32.2	9.9	7.4	5.9 ↓	3.00E-07	7.82E-05
hsa-miR-125b	11q24.1/21q11.2	11.4	8.9	5.8 ↓	6.00E-07	7.82E-05
hsa-miR-99a	21q11.2	7.4	6.3	2.2 ↓	1.30E-06	0.0001
hsa-miR-100	11q24.1	7.6	6.4	2.2 ↓	2.00E-06	0.0001
hsa-miR-145	5q32-33	7.3	6.4	1.8 ↓	4.95E-05	0.0024
hsa-miR-195	17p13	9.1	7.4	3.4 ↓	0.000152	0.0062
hsa-miR-10b	2q31	7.6	6.8	1.7 ↓	0.00024	0.0084
hsa-miR-320c	18q11.2	7.6	6.9	1.6 ↓	0.000412	0.0127
hsa-miR-320d	13q14.11/Xq27.1	7.4	6.7	1.6 ↓	0.000512	0.014
hsa-miR-101	1p31.3	7.4	6.4	2.0 ↓	0.000787	0.0185
hsa-miR-130a	11q12	7.5	6.8	1.6 ↓	0.000887	0.0185
hsa-miR-320b	1p13.1/1q42.11	8.1	7.3	1.8 ↓	0.000938	0.0185
hsa-miR-125a-5p	19q13.4	8.8	8.1	1.6 ↓	0.000976	0.0185
hsa-miR-335	7q32.2	7.1	6.5	1.4 ↓	0.001195	0.021
hsa-miR-320a	8p21.3	8.1	7.3	1.8 ↓	0.001669	0.0257
hsa-miR-143	5q32-33	9.7	8.2	2.8 ↓	0.002078	0.0301
hsa-miR-21	17q23.1	9.1	10.1	2.0 ↑	0.003822	0.0495
hsa-miR-30c	6q13	8.4	7.8	1.5 ↓	0.004283	0.0504
hsa-miR-300	14q32.31	7.1	8	2.0 ↑	0.004306	0.0504

Supervised clustering over the 19 differentially expressed miRNAs in normal breast tissue, tumor tissue and normal Human Mammary Epithelial Cells (HMEC) is shown in **Figure 21**. Expression of these 19 differentially expressed miRNAs in immortalized but not transformed HMEC cells was similar to the expression in normal breast tissue although some differences existed for specific miRNAs. HMEC cells represent normal proliferating cells while normal breast tissue represent preferentially non proliferating cells, suggesting that down regulation of some miRNAs, such as miR-21, miR-99a, miR-101, miR-143 or miR-145, might be related to proliferation in both normal and tumoral cells. However, down-regulation of other miRNAs, miR-205, miR-125a/b, miR-100 or miR-30c, could have a role in more specific tumoral processes.

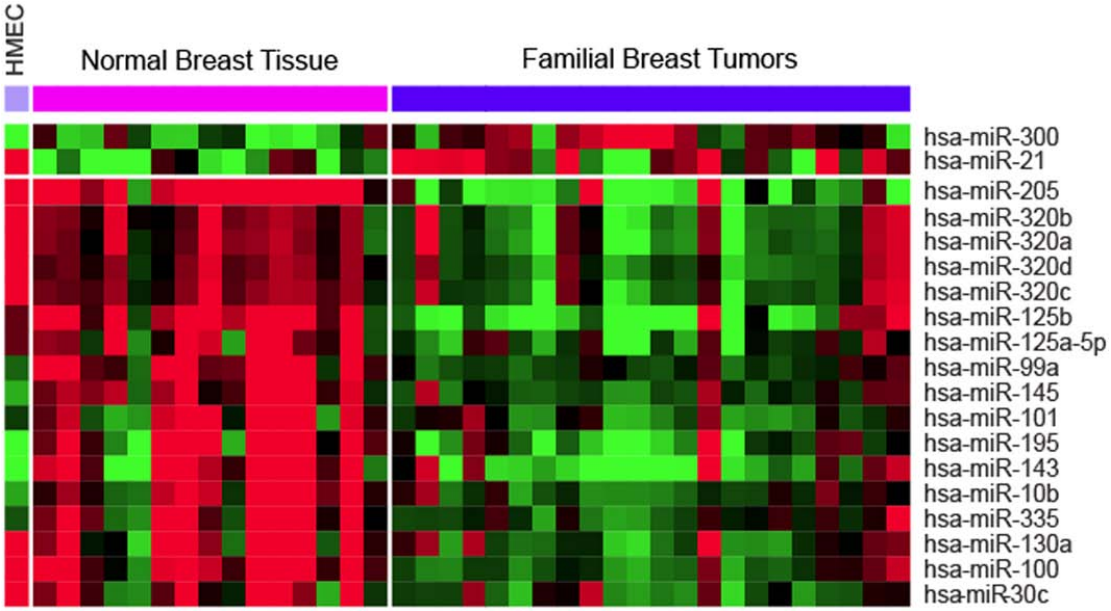


Figure 21. Heat map showing 19 differentially expressed miRNAs between normal breast samples and hereditary breast tumors; expression higher than median in red, lower in green. Expression of these miRNAs in Human Mammary Epithelial Cells (HMEC) cells is also represented.

2.2. miRNAs commonly deregulated in hereditary and sporadic breast cancer

Interestingly, several miRNAs that we found to be deregulated in hereditary breast cancer were previously described to be deregulated in sporadic breast tumors. Thus, 11 of the 19 miRNAs (miR-10b, -100, -101, -125a, -125b, 130a, -143, -145, -21, -205, and -30c) were previously identified in two key studies as being aberrantly expressed in sporadic breast tumors in comparison to normal tissue (Iorio et al., 2005; Volinia et al., 2006), suggesting that these miRNAs may play a general role in breast carcinogenesis (**Figure 22**). Deregulation of some of these miRNAs in sporadic tumors was also confirmed by qRT-PCR analysis in an independent set of 18 sporadic breast tumors (**Figure 23**).

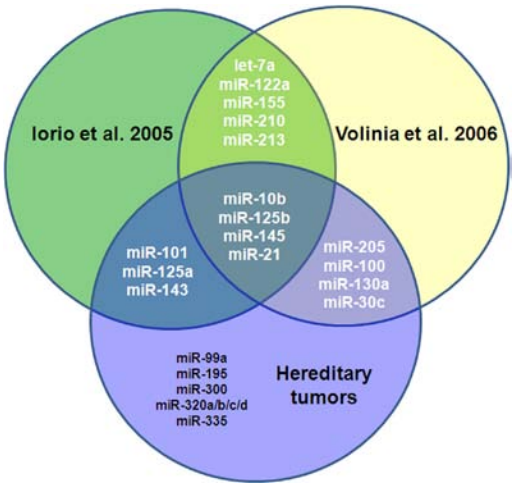


Figure 22. Venn diagrams representing commonly deregulated miRNAs in two different studies carried out in sporadic breast cancer samples and in the present study on hereditary breast tumors. Regardless of the genetic background or histopathological features of the tumors, there are miRNAs consistently altered in breast tumor samples in comparison to normal breast tissue.

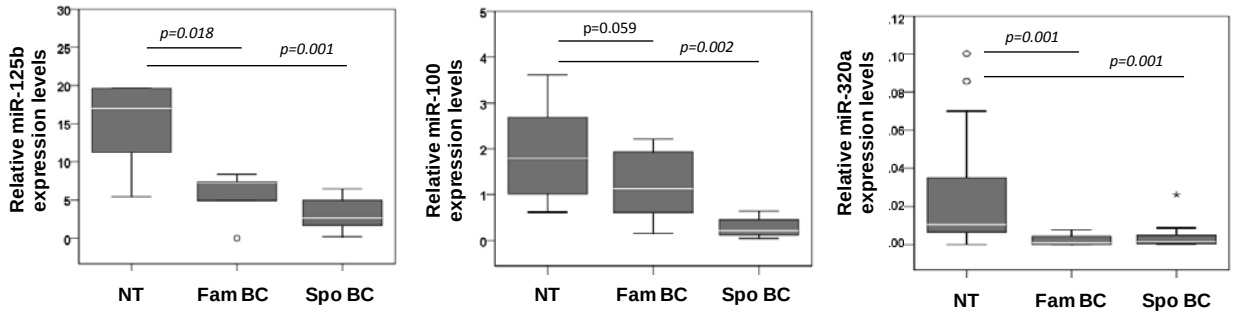


Figure 23. Validation of miRNA expression by qRT-PCR in hereditary and sporadic tumors. Expression levels of miR-125b, miR-100, miR-320a in normal breast tissue comparing with hereditary tumor samples (FamBC) and sporadic breast cancer (SpoBC). Differences were estimated by t-test and *p* values are shown for each case.

2.3. Pathway enrichment analysis

Given the fact that a single miRNA can target a large number of mRNA transcripts, aberrant expression of a set of miRNAs could have significant effect on cellular function by affecting multiple signaling pathways. To assess the potential impact of deregulated miRNA in hereditary breast tumors on biological processes and pathways, we used Diana miRPath web-based computational tool for biological interpretation of miRNA profiling data using over-representation analysis of biological processes and signaling pathways that are targeted collectively by co-expressed miRNAs. KEGG pathway enrichment analysis has revealed that the set of differentially expressed miRNAs between normal tissues and hereditary breast tumors, targets multiple effectors of pathways involved in ubiquitylation, cell proliferation and migration. Full list of pathways that have significantly overrepresented genes ($p<0.05$) collectively targeted by the set of 19 differentially expressed miRNAs is shown in **Table 4**. Observed down regulation of miRNAs in tumors regulating expression of these genes may result in abnormally activated pathways leading to increased proliferation and/or migration abilities.

Table 4. Significantly enriched signaling pathways associated to the differentially expressed miRNAs

KEGG Pathway	Nº of miRNA Target Genes	-ln(p-value)	Gene Names
Ubiquitin mediated proteolysis	20	17.18	UBE2D1, SOCS1, UBE2D2, UBE3C, UBE1, MAP3K1, BIRC6, UBE2J1, UBE2I, SMURF1, UBE2W, CBL, BTRC, WWP1, CUL2, SOCS3, CBLB, NEDD4L, NEDD4, ITCH
Axon guidance	17	11.88	SRGAP3, PLXNA2, GNAI2, DPYSL2, ITGB1, UNC5C, EFNA3, SEMA4D, EPHB2, SEMA6D, KRAS, CFL2, NRP1, PPP3CA, RASA1, SEMA3A, NFAT5
Insulin signaling pathway	16	7.96	PPARGC1A, SOCS1, MAPK8, TSC1, PDPK1, KRAS, CBL, FRAP1, CRKL, SORBS1, SOS1, FOXO1, SOCS3, CBLB, PIK3CD, AKT3
O-Glycan biosynthesis	6	7.23	GALNT2, GALNT7, GALNT1, GALNT3, GCNT1, B4GALT5
mTOR signaling pathway	7	5.31	TSC1, PDPK1, FRAP1, ENSG00000164327, RPS6KA3, PIK3CD, AKT3
ErbB signaling pathway	10	4.74	MAPK8, NRG3, KRAS, CBL, FRAP1, CRKL, SOS1, CBLB, PIK3CD, AKT3
Glycan structures - biosynthesis 1	12	4.35	GALNT2, GALNT7, GALNT1, GALNT3, CHST2, STT3B, CHST1, GCNT1, EXTL2, B4GALT5, MAN1A2, XYLT1
MAPK signaling pathway	20	3.84	MAP4K4, MAPK8, MAP3K1, KRAS, MEF2C, BDNF, CRKL, TAOK1, STK4, SOS1, FGFR1, RPS6KA5, FGF2, RAP1B, PPP3CA, NF1, RASA1, MAP3K12, RPS6KA3, AKT3
Regulation of actin cytoskeleton	17	3.61	ITGB1, WASL, ARHGEF6, KRAS, PIP4K2B, CRKL, CFL2, PIP4K2A, ITGA6, SOS1, FGFR1, FGF2, ITGB3, GNA13, ACTC1, PIK3CD, PFN2
Adherents junction	8	3.55	IGF1R, SNAI1, WASL, SMAD2, SORBS1, FGFR1, SSX2IP, PVRL1
Focal adhesion	16	3.54	BCL2, MAPK8, ITGB1, IGF1R, PDPK1, CRKL, ITGA6, SOS1, PTEN,PTENP1, RAP1B, ITGB3, ARHGAP5, CCND2, PIK3CD, AKT3, CCND1
T cell receptor signaling pathway	9	3.2	KRAS, CBL, SOS1, PPP3CA, CBLB, PDCD1, NFAT5, PIK3CD, AKT3

We focused our further analysis on the MAPK signaling pathway since a large number of genes within this pathway were found to be commonly targeted by 8 out of 19 deregulated miRNAs in our study suggesting that these miRNAs might cooperate to affect gene expression and consequentially activation or repression of signaling pathways (Table 5). Interestingly, miR-30c has potential binding sites on 12 different target genes involved in MAPK pathway. This miRNA potentially targets important mediators of MAPK signaling, such as KRAS, RASA1, MAP3K1 and MAPK8. Furthermore, KRAS gene has been previously validated as a target of several miRNAs, including let-7, miR-143 and miR-145 previously found to be significantly down-regulated in breast tumors (Chen et al., 2009; Johnson et al., 2005; Yu et al., 2010b). Therefore, we investigated whether miR-30c could be regulating KRAS expression in hereditary breast tumors. Thus, we confirmed by QRT-PCR that miR-30c had decreased expression in both hereditary and sporadic breast tumors comparing to normal samples (Figure 24). Although mutations in KRAS are infrequent in breast tumors, activation of KRAS pathway in breast cancer have been frequently found. All these data suggests that deregulation of miRNAs could represent a potential mechanism to explain KRAS overexpression in breast tumors.

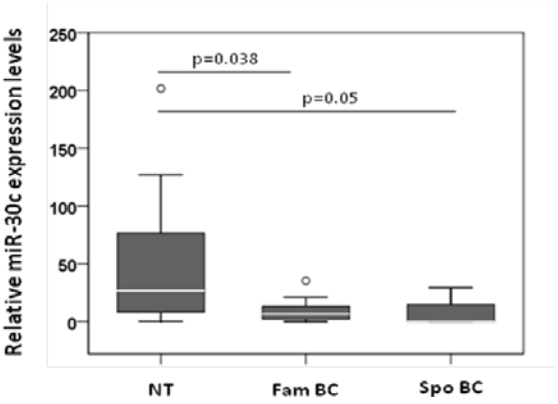


Figure 24. Validation of miR-30c expression by qRT-PCR in an independent set of 12 hereditary (FamBC) and 8 sporadic breast tumors (SpoBC) comparing to normal breast tissue expression. Differences were estimated by t-test and *p* values are shown for each comparison.

Table 5. Genes within MAPK pathway predicted by DIANA microT 4.0 to be targeted by deregulated miRNAs in hereditary breast cancer tumors.

miRNA Name	Gene	Gene Definition
hsa-miR-205	RPS6KA3	ribosomal protein S6 kinase, 90kDa, polypeptide 6
hsa-miR-99a	PPP3CA	calcium binding protein P22
	FGFR1	fibroblast growth factor receptor 1
hsa-miR-100	PPP3CA	calcium binding protein P22
	FGFR1	fibroblast growth factor receptor 1
hsa-miR-195	FGF2	fibroblast growth factor 2
	MAPK8	mitogen-activated protein kinase 8
	AKT3	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)
hsa-miR-101	RAP1B	RAP1A, member of RAS oncogene family
	STK4	serine/threonine kinase 3
hsa-miR-130a	RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 6
hsa-miR-300	PPP3CA	calcium binding protein P22
	MEF2C	myocyte enhancer factor 2C
hsa-miR-30c	SOS1	son of sevenless homolog 1 (Drosophila)
	KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
	NF1	neurofibromin 1
	RASA1	RAS p21 protein activator (GTPase activating protein) 1
	RAP1B	RAP1A, member of RAS oncogene family
	PPP3CA	calcium binding protein P22
	MAPK8	mitogen-activated protein kinase 8
	MAP4K4	mitogen-activated protein kinase kinase kinase kinase 4
	MAP3K1	mitogen-activated protein kinase kinase kinase 1
	TAOK1	TAO kinase 3
	BDNF	brain-derived neurotrophic factor
	CRKL	v-crk sarcoma virus CT10 oncogene homolog (avian)

2.4. miR-30c regulates KRAS expression

The role of miR-143 and miR-145 in regulating KRAS expression was already described; however KRAS was not previously reported as miR-30c target. Therfore, we explored the role of miR-30c in regulation of KRAS expression. We observed a negative correlation between miR-30c expression and KRAS protein level in two breast cancer cell lines (**Figure 25**). In addition, several bioinformatic target prediction algorithms (DIANA microT4.0, TargetScan, PITA, PicTar, miRANDA) indicated existence of a broadly conserved putative binding site for miR-30c in the 3'UTR of the KRAS gene (**Figure 26A**). To test the hypothesis that KRAS is a bona fide target of miR-30c, we constructed a reporter plasmid harboring 300bp of the wild-type 3'UTR region of KRAS flanking miR-30c binding site downstream of the luciferase coding region. MDA-MB-436 cells were co-transfected with luciferase reporter and pre-miR-30c or scramble control. As a result, pre-miR-30c transfected cells showed a marked reduction (52%) of luciferase activity compared to scramble control, confirming the interaction between miR-30c and KRAS 3'UTR binding site (**Figure 26B**). Next, we checked whether miR-30c has an effect on KRAS mRNA stability by performing qRT-PCR analysis in MDA-MB-436 cells transiently transfected with either pre-miR-30c or scramble control. Indeed, we observed a sharp decrease in KRAS mRNA levels upon transfections with pre-miR-30c (**Figure 26C**). In addition, pre-miR-30c or pre-miR- control were transfected into MDA-MB-436 cells, and we confirmed a reduction of about 44% of KRAS protein level in MDA-MB-436 cells over-expressing miR-30c, in comparison to control (**Figure 26D**).

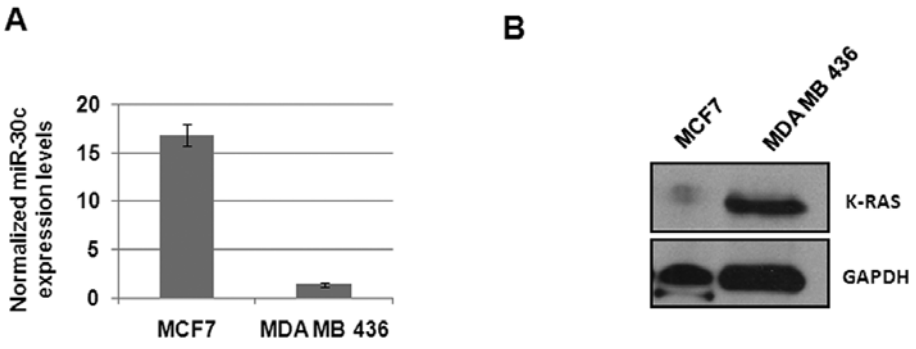


Figure 25. Correlation of expression of miR-30c and KRAS in two breast cancer cell lines. Inverse correlation between the expression level of miR-30 determine by qRT-PCR (A) and detection of KRAS protein in MCF7 and MDA-MB-436 cells (B).

2.5. miR-30c affects breast cancer cell proliferation

To gain more insight into the biological effects of miR-30c on breast tumorigenesis given that KRAS plays a role in regulation of cell proliferation, we transfected MDA-MB-436 cells, which previously showed elevated levels of KRAS protein, with pre-miR-30c or scramble control and analyzed the cell growth by MTT assay. As shown in Figure 15E, ectopic expression of miR-30c resulted in reduced proliferation in comparison to scramble control transfected cells. Therefore, modulation of KRAS protein level by miR-30c may explain at least in part, how down-regulation of miR-30c can promote proliferation and contribute to tumorigenesis.

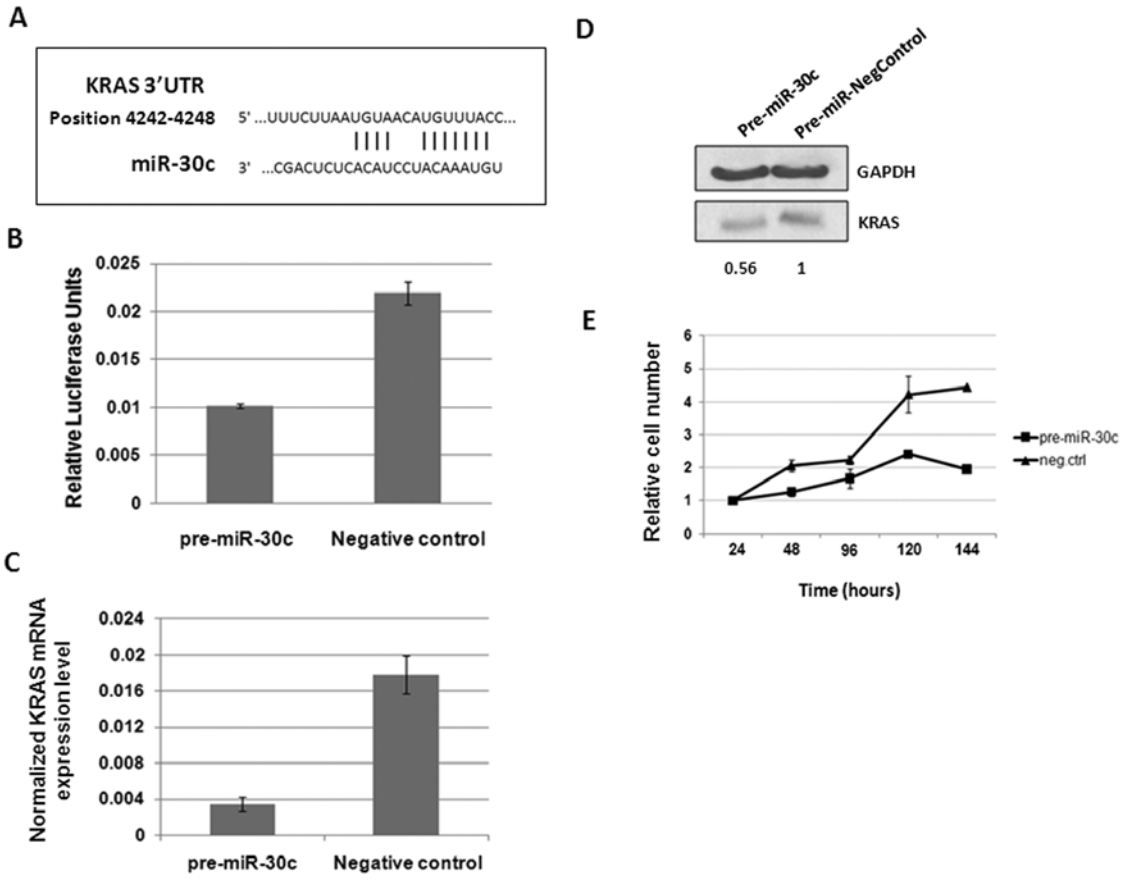


Figure 26. miR-30c effects on KRAS expression and cell proliferation. **A)** Schematic representation of miR-30c binding site within the KRAS 3'UTR region. **B)** Luciferase activity of a reporter construct carrying the KRAS 3'UTR downstream of the luciferase gene. The construct was co-transfected with pre-miR-30c or scramble control in MDA-MB-436 cells. **C)** KRAS expression at transcription level. Significant reduced level of KRAS mRNA expression was detected by qRT-PCR after pre-miR-30c transfection, comparing with scramble control. **D)** Regulation of KRAS protein level by miR-30c. MDA-MB-436 cells were transfected with pre-miR-30c or

pre-miR-scramble oligonucleotides. After 48 hours KRAS protein was evaluated by western blot. GAPDH was used as loading control. The signal in each line was quantified and the ratio of KRAS to GAPDH was determined. **E)** Effect of miR-30c expression on proliferation of MDA-MB-436 cells. MTT cell viability assay was performed at 48, 72, 96, 120 or 144 hours after transfection of MDA-MB-436 cells with pre-miR-30c or pre-miR-scramble oligonucleotides.

RESULTS PART III

3. MIRNA EXPRESSION PROFILING OF BREAST TISSUE SAMPLES

The objective of the third part of the study was to characterize hereditary breast tumors based on their miRNA expression profiles. To this end, we performed global miRNA expression analysis of more than 800 human miRNA genes in a large series of 80 FFPE breast tissue samples. The series included 66 hereditary breast primary tumors from 13 BRCA1 mutation carriers, 10 BRCA2 mutation carriers and 43 non-BRCA12 tumors denominated hereafter as BRCAX tumors. In addition we have analyzed 10 sporadic breast carcinomas and 6 normal breast tissues obtained after breast reduction surgery from healthy donors with no family history of breast cancer. To avoid contamination with normal breast tissue, tumoral area on FFPE blocks was marked by a pathologist and macrodissected for subsequent total RNA extraction. Histopathological characteristics (ER, PR, HER2, EGFR, CK5, Ki-67), tumor subtype and age at diagnosis of BRCA1, BRCA2 and BRCAX tumors in this series (**Table 6**) are fully representative to what has been reported in literature. However our series has higher proportion of high grade tumors among BRCAX cases to what has been reported previously, potentially due to stringent ascertainment criteria for selection of hereditary breast tumor applied in this series.

3.1. Unsupervised hierarchical clustering of all breast tissue samples

Specific miRNA profiles have been associated to different tumor types (Lu et al., 2005) and it has been documented that miRNAs can discriminate between molecular subgroups and histopathological features in sporadic breast cancer. To assess whether there are differences in miRNA expression profiles between different groups, namely BRCA1 and BRCA2 mutated and non-BRCA1/2 hereditary breast tumors, sporadic breast carcinomas and normal breast tissue, we performed an unsupervised hierarchical clustering analysis. After removing gene patterns containing missing values, an additional filter procedure was applied eliminating miRNAs with uniformly low expression ($\log_2$<6.5) or with low expression variation across the experiments (V<0.1), retaining 444 miRNA genes. Unsupervised hierarchical clustering over 444 miRNAs, applied to the whole series of breast tissues demonstrated clear separation of different tumor groups. Strikingly, BRCA1 and BRCA2 tumors were clustered together in a homogenous group separated from BRCAX and sporadic tumors, and apart from normal breast tissue. Furthermore, both sporadic breast tumors and normal breast tissues were maintained as two homogenous groups, while BRCAX tumors exhibited heterogeneous miRNA expression profiles (**Figure 27)**.

Table 6. Summary of clinico-pathological characteristics for familial breast tumors

	BRCA1		BRCA2		BRCAX	
Total No.	**n=13**		**n=10**		**n=43**	
	n	%	n	%	n	%
Age at diagnosis	**9**		**6**		**40**	
mean	40.33		42.5		47.75	
range	28-55		35-56		25-95	
Lymph Node	**13**		**10**		**37**	
positive	5	50%	4	57.10%	18	48.60%
negative	5	50%	3	42.90%	17	51.40%
Grade	**12**		**10**		**43**	
1	0	0%	2	20%	4	9.30%
2	1	8.30%	3	30%	20	48.80%
3	11	91.70%	5	50%	17	41.50%
Estrogen Receptor	**13**		**10**		**37**	
positive	2	15.40%	7	70%	18	48.60%
negative	11	84.60%	3	30%	19	51.40%
Progesteron Receptor	**12**		**10**		**38**	
positive	2	15.40%	7	70%	14	36.80%
negative	11	84.60%	3	30%	24	63.20%
HER2	**13**		**10**		**38**	
positive	0	0%	2	20%	9	23.70%
negative	13	100%	8	80%	29	76.30%
EGFR	**13**		**8**		**38**	
positive	6	46.20%	0	20%	1	23.70%
negative	7	53.80%	8	100%	37	97.40%
p53	**12**		**6**		**31**	
positive	7	58.30%	2	33.30%	10	32.30%
negative	5	41.70%	4	66.70%	21	67.70%
CK5	**13**		**10**		**38**	
positive	9	69.20%	1	14.30%	4	14.80%
negative	4	30.80%	6	85.70%	23	85.20%
Ki-67	**13**		**7**		**31**	
1 0-5%	4	30.80%	3	42.90%	15	48.30%
2 6-25%	5	23.10%	2	28.60%	11	35.50%
3 >25%	4	30.80%	2	28.60%	5	21.60%
Subtype	**13**		**10**		**35**	
Luminal A	2	15.40%	6	60%	13	37.10%
Luminal B	0	0%	2	20%	6	17.10%
HER2	0	0%	0	0%	3	8.60%
Triple Negative	11	84.60%	2	20%	13	37.10%

Breast cancer cases were classified into four subtypes based on IHC-model Tang P. et al, 2009.

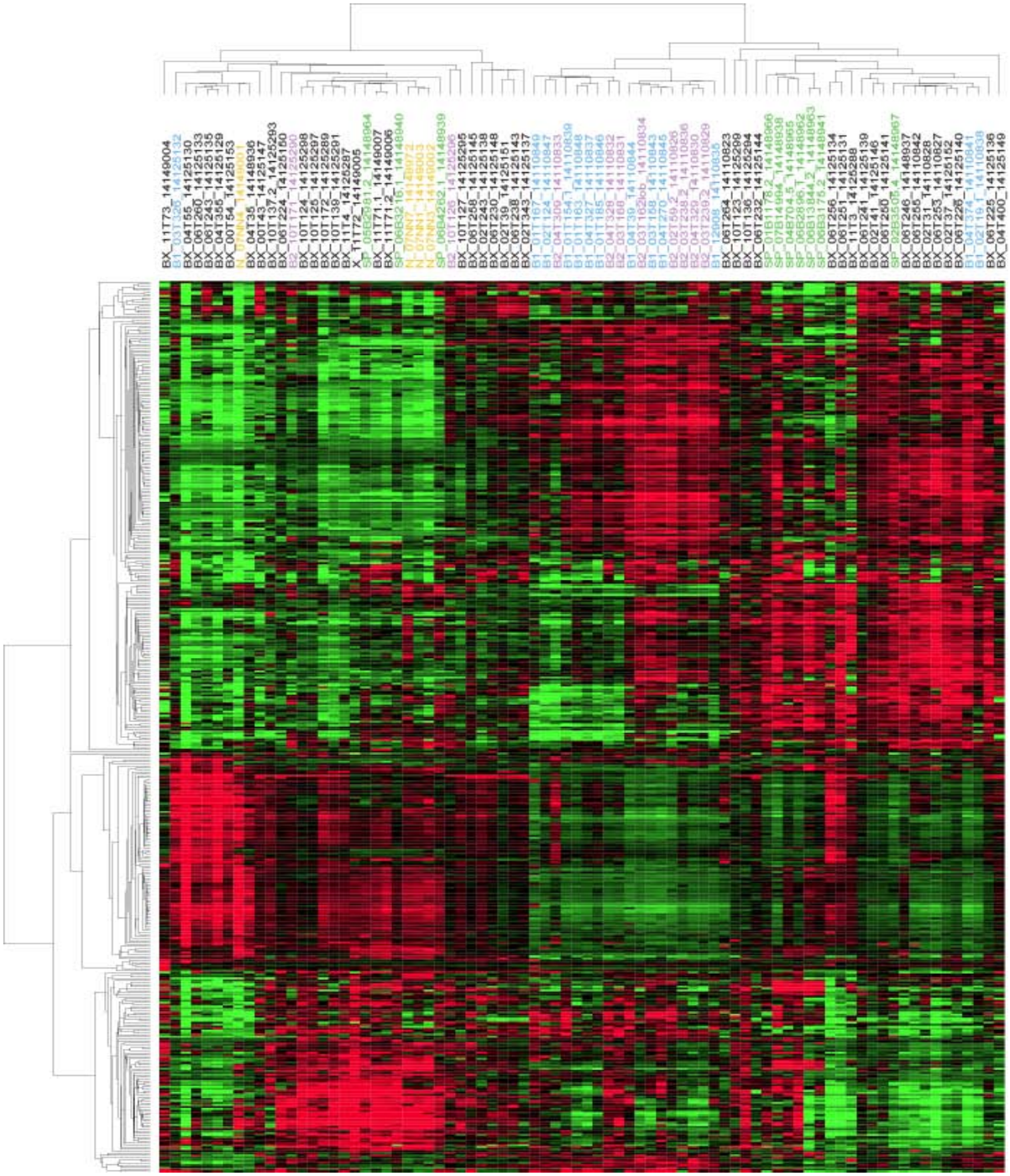


Figure 27. Heatmap showing unsupervised hierarchical clustering (average linkage clustering, Pearson correlation coefficient, median centered) of 80 FFPE breast samples over 444 miRNAs. BRCA1-mutated samples are marked in *blue*, BRCA2-mutated in *purple*, BRCAX in *black*, sporadic breast tumors in *green* and normal breast tissues in *yellow*. Heatmap is representing miRNA expression in red-black green pseudo scale.

3.2. miRNA molecular profiles in hereditary breast tumors

Unsupervised hierarchical clustering of 66 hereditary breast tumors (**Figure 28**) was performed over 444 miRNAs that were retained after filtering out miRNAs with low expression variation (VAR <0.1) and low expression (log_2 <6.5). Clustering analysis revealed very heterogeneous miRNA profiles among hereditary breast tumors that were separated in two main branches with contrasting miRNA expression profiles. This bi-partition correlates with the tumor grade with the left branch, consisted almost exclusively of BRCAX tumors, being predominantly low and moderate grade tumors, while the right branch, is consisted of mainly high grade tumors. The right branch was further partitioned in two sub-branches; one consisted exclusively of BRCA1 and BRCA2 tumors, and the other mainly of BRCAX tumors and two BRCA1 samples. It is been well documented that breast tumors can be classified into four stable molecular subtypes by mRNA profiling, with the most prominent discriminators being ER, HER2 and tumor differentiation. Interestingly, the partitioning based on miRNA expression is not mainly driven by ER status. We observed clustering of HER2 positive tumors, while triple negative (ER-, PR- and HER-) tumors were dispersed across the cluster.

Unsupervised clustering of hereditary breast tumors over 444 miRNA genes (v>0.1) showed that BRCA1 and BRCA2 tumors clustered in the same sub-branch representing a homogenous group with very similar miRNA expression profiles, and clearly separated from the BRCAX tumors. Of note, two out of tree BRCA1 outliers were ER positive tumors and both BRCA2 tumors that do not cluster in the "BRCA" group were the only two of luminal B subtype (ER+, PR+, HER2+). BRCAX tumors were separated into two branches with heterogeneous miRNA expression profiles that demarcate several subgroups. One group of BRCAX samples was clustering along BRCA1 and BRCA2 mutated tumors, while samples in other branch had markedly different miRNA expression profile.

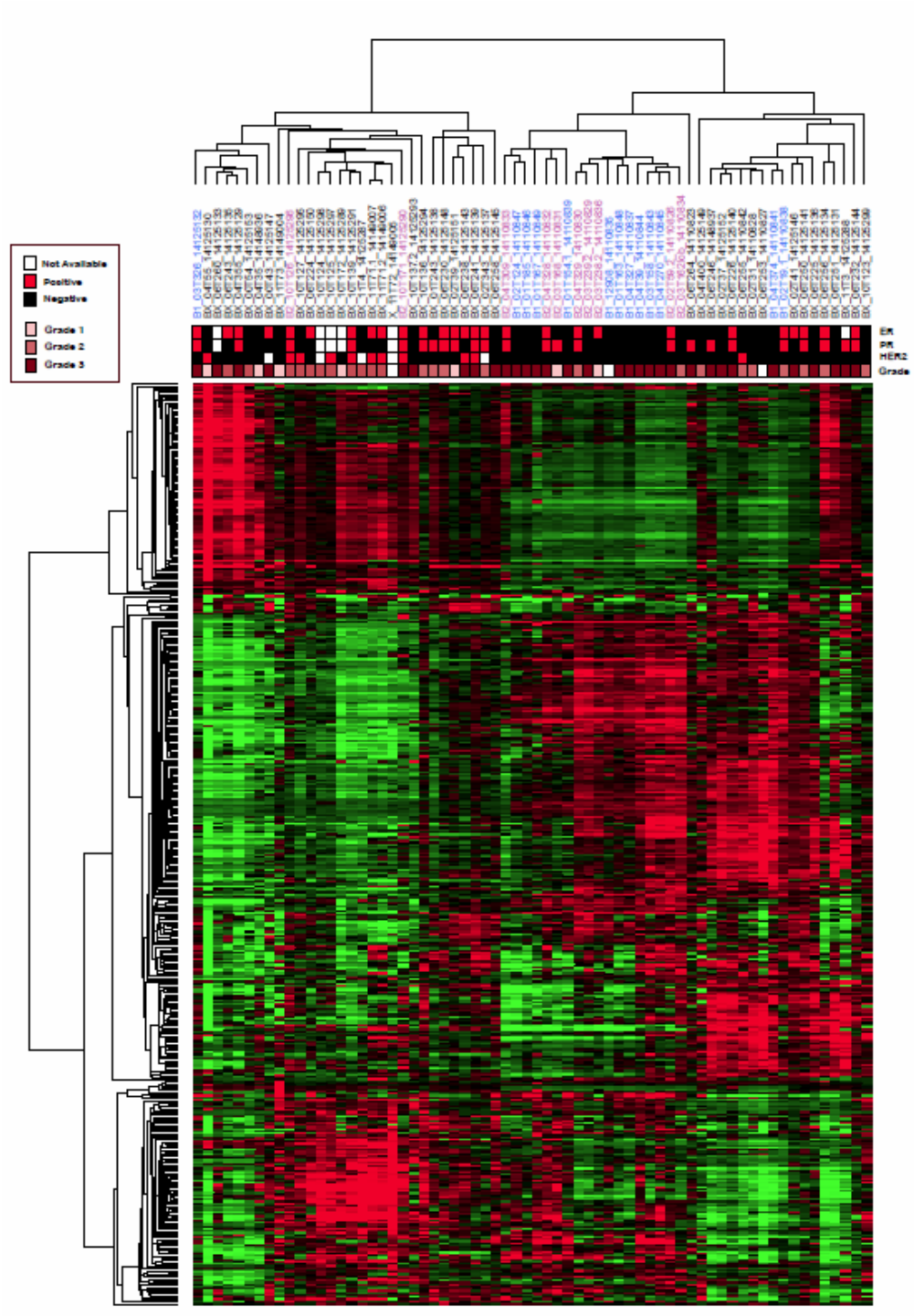


Figure 28. Unsupervised hierarchical clustering (average linkage clustering, Pearson correlation coefficient, median centered) of 66 hereditary breast tumors including 13 BRCA1 (blue), 10 BRCA2 (purple) and 43 BRCAX (black) over 444 miRNAs (v>0.1). Immunohistochemistry markers (pathology report or tissue microarray) for ER, PR and HER2, as well as tumor grade are represented. In such cases where the pathology report and TMA results were discrepant, the pathology report was used.

3.3. miRNAs differentially expressed between breast tumor groups

In order to find miRNAs discriminating between different genetic subgroups we performed differential expression analysis applying limma t-test, with significance threshold set to 5% after correction for multiple testing by Benjamini and Hoceberg method. The analysis revealed a large number of differentially expressed miRNAs between most classes compared. The results are summarized in **Table 7**. Importantly, a large number of miRNAs (151) was found to be differentially expressed between BRCA1/2 mutated tumors and both BRCAX and sporadic breast tumors, as well as between BRCA1/2 mutated and BRCAX tumors (150), or sporadic (138), respectively. Surprisingly, no miRNA genes were significantly differentially expressed between BRCA1 and BRCA2 mutated tumors, in contrast to what has been observed by gene expression profiling [13]. Only two miRNAs, miR-125a and miR-7, had significant unadjusted p-values, but after correction for multiple testing this significance was lost. Hereditary tumors a whole had much less differentially expressed miRNAs (38). In addition; only 22 miRNAs were differentially expressed between BRCAX hereditary breast tumors and sporadic tumors, suggesting higher similarity of miRNA expression profiles between BRCAX and sporadic breast tumors. However, due to high heterogeneity of BRCAX tumors some differences might be hindered in the analysis.

Table 7. Summary of differentially expressed miRNAs (FDR adjusted p-value <0.05) by limma t-test analysis between breast carcinomas with different BRCA1/2 status

TUMOR TYPE	TUMOR TYPE COMPARED TO	Upregulated miRNAs	Downregulated miRNAs	Total differentially expressed miRNAs
BRCA1 and BRCA2	BRCAX	63	87	150
BRCA1	BRCAX	55	71	126
BRCA2	BRCAX	38	41	79
BRCA1	BRCA2	0	0*	*2 miRNAs with unadjusted p-value <0.05
HEREDITARY	SPORADIC	6	32	38
BRCA1 and BRCA2	SPORADIC	44	94	138
BRCA1	SPORADIC	43	82	125
BRCA2	SPORADIC	36	67	103
BRCAX	SPORADIC	2	20	22
BRCA1 and BRCA2	BRCAX and SPORADIC	62	89	151

Among miRNAs differentiating between BRCA1/2, BRCAX and sporadic tumors, large fraction of miRNAs exhibited a gradual change in their expression levels between the groups, in the formerly specified order. This was manifested in a large overlap between miRNAs differentiating between BRCA1/2 tumors *vs*. both BRCAX and sporadic, or just BRCAX tumors as represented in the Venn diagram (**Figure 29**), and those differentiating BRCAX tumors from sporadic ones, indicating that only few miRNAs can serve as specific markers for each subgroup.

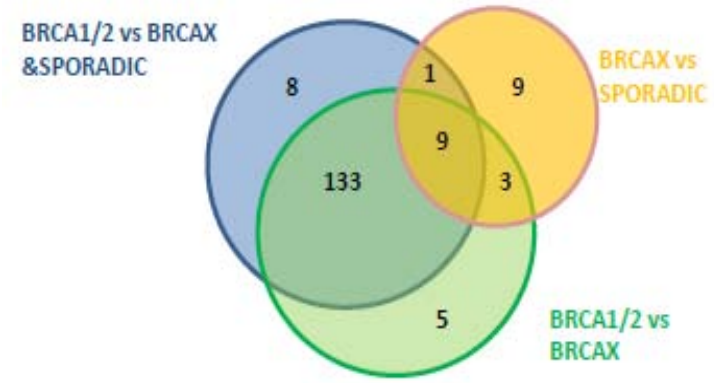


Figure 29.Venn diagram representing overlap of miRNA genes differentially expressed between different subtypes of breast tumors. miRNAs differentially expressed between BRCA1/2 tumors vs. both BRVCAX and sporadic breast tumors are represented in blue circle, those differentially expressed between BRCA1/2 tumors and BRCAX are in green, and the ones differentially expressed between BRCAX tumors and sporadic breast carcinomas in yellow.

Supervised clustering over 151 miRNA genes found to be differentially expressed between tumors harboring mutation in either BRCA1 or BRCA2 gene, and non-BRCA1/2 and sporadic tumors is represented in Figure 18. Among 151 miRNAs that were found to be differentially expressed between tumors from BRCA mutation-carriers versus non-carriers, 89 miRNAs were downregulated and 62 were upregulated in BRCA1/2 tumors, out of which 5 miRNAs were downregulated and 2 upregulated with higher than 2 fold-change differences in expression.

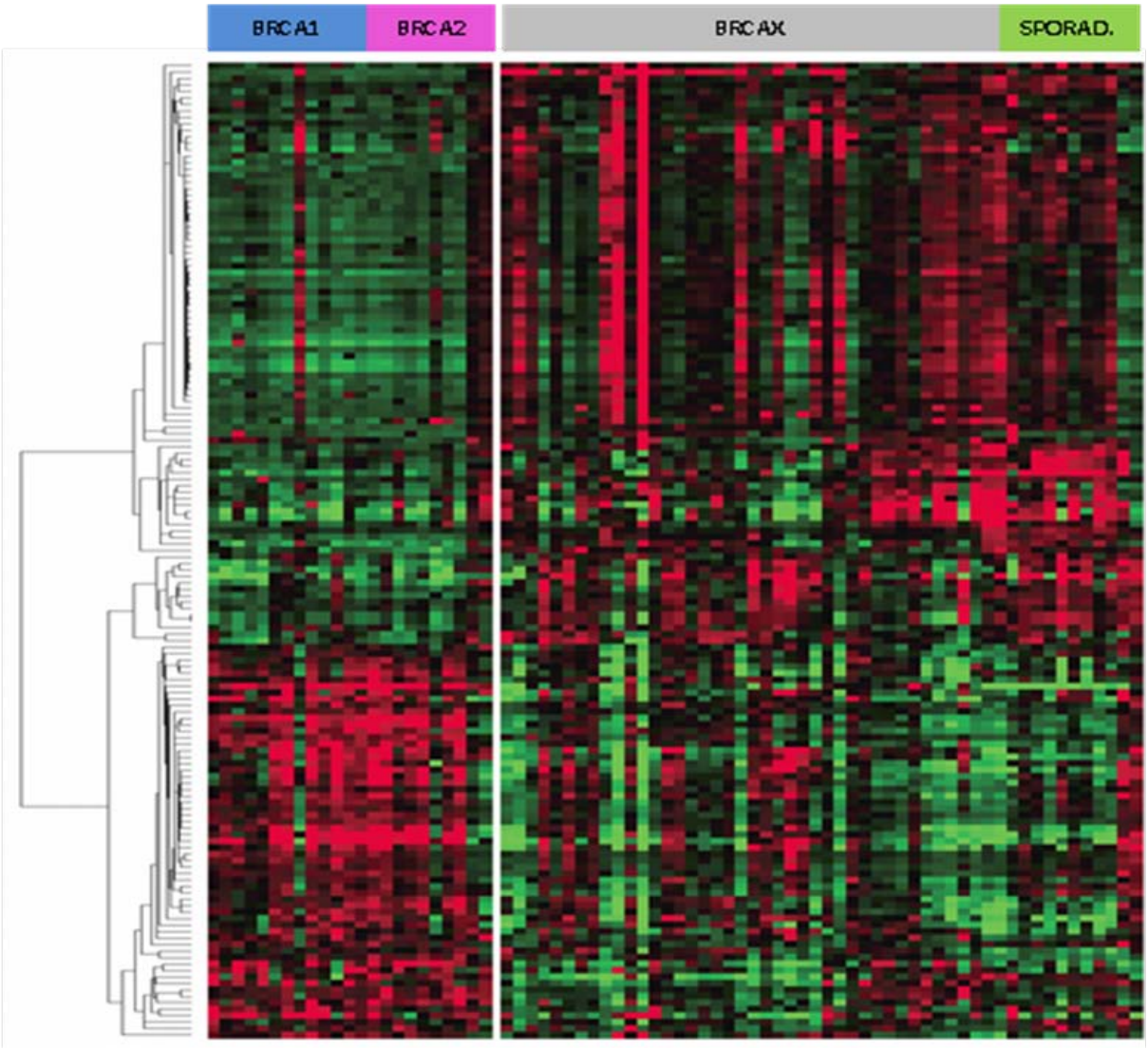


Figure 30.Supervised clustering of breast tumors over 151 miRNAs differentiating between BRCA1 and BRCA2 tumors *versus* BRCAX and sporadic breast tumors. Heatmap representing median centered miRNA gene expression in rows, red indicating higher green lower than median expression. Samples are represented in columns, BRCA1 tumors in blue, BRCA2 in pink, BRCAX in gray and sporadic in green.

3.4. Class discovery algorithms reveal subgroups of BRCAX tumors

In order to gain more insight into intrinsic characteristics of BRCAX tumors we performed unsupervised hierarchical clustering of 43 BRCAX tumor samples over 444 miRNA genes (v>0.1) that revealed clear heterogeneity in their miRNA expression profiles (**Figure 31A**) with several apparent subgroups. Consensus clustering (GenePattern 2.0, Broad Institute) bioinformatic tool has been used for class discovery to obtain the most robust classification of BRCAX tumors, by reiteration of a selected clustering algorithm (KNN means with 2,3,4,5 centroids) and by assessing the stability of discovered clusters. This analysis indicated the existence of 4 different subgroups among BRCAX tumors, namely BRCAX- A, -B, -C and -D (**Figure 31B and C**).

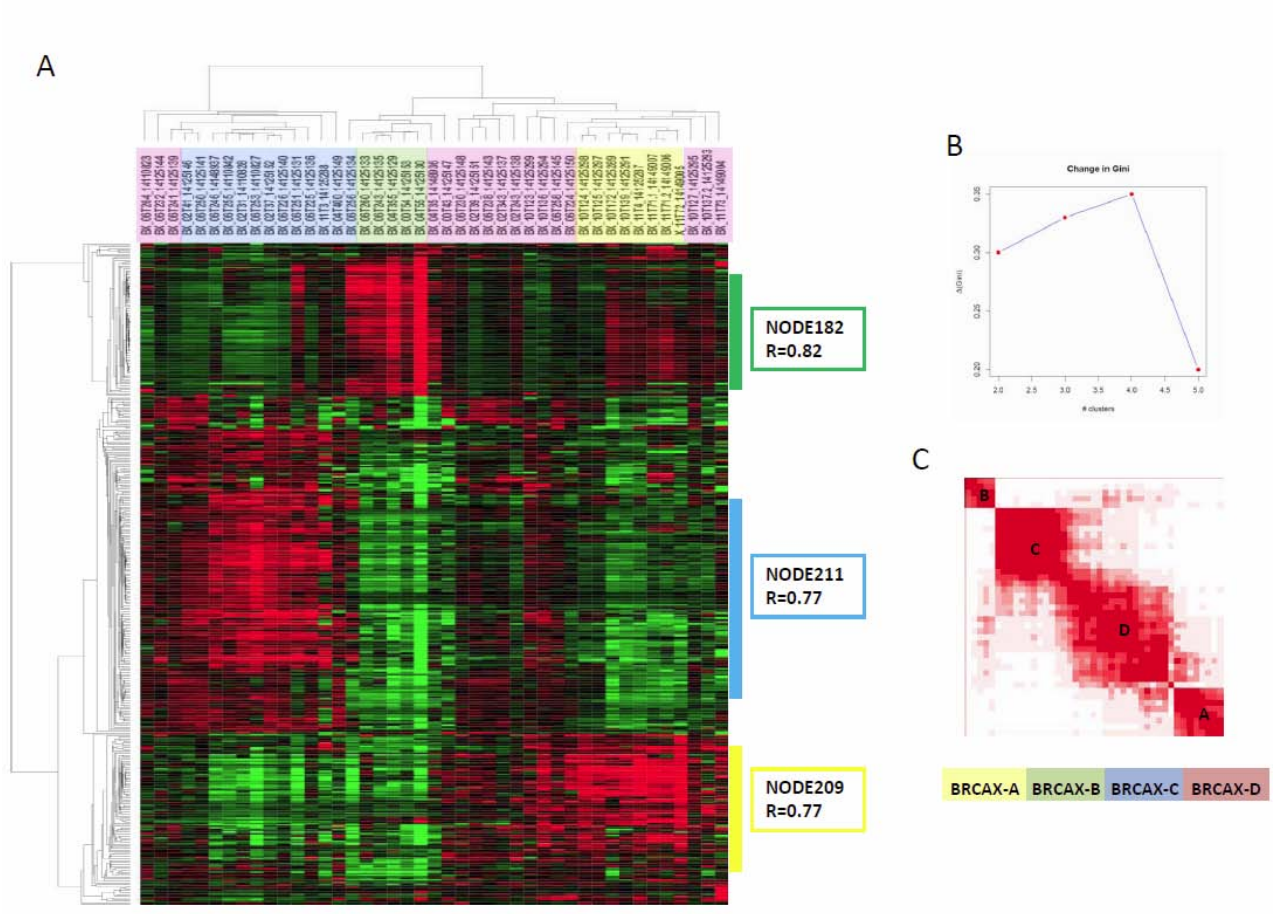


Figure 31. A) Unsupervised hierarchical clustering of 43 BRCAX tumors over 444 miRNAs (v>0.1). **B)** Resampling of the BRCAX series by Consensus Clustering algorithm (KNN Means, Euclidian distance). Plot is showing the change in free energy (ΔGn) with each additional subgroup added, indicating that optimal number of clusters within this series is four. C) Red squares in the consensus matrix are representing subgroups of BRCAX tumors in which the samples were more robustly distributed based on their miRNA expression profile. Four subgroups of tumors were identified and denominated as BRCAX-A (yellow), BRCAX-B (green), BRCAX-C (blue) and BRCAX-D (pink).

3.5. miRNA signatures defining BRCAX subgroups

To further characterize BRCAX subgroups we have selected co-expressed miRNAs with correlation coefficient higher than R^2>0.77 that best discriminate between the groups (**Figure 31 A**). Three main miRNA clusters were identified namely, Cluster 1 consisting of 62 miRNAs, Cluster 2 consisting of 48 miRNAs, and Cluster 3 with 73 miRNAs. Full list of miRNA genes within each cluster and their average values in each tumor group are shown in **Supplementary Tables 5,6 and 7**.

Supervised clustering of 43 BRCAX tumors according to their subgroup, 23 BRCA1 and BRCA2 mutated tumors, 10 sporadic breast tumors and 10 normal breast tissue samples over 180 miRNAs belonging to miRNA Clusters 1, 2 and 3 is represented in **Figure 32**. By visual inspection we observed lower expression of Cluster 1 and Cluster 2 miRNAs in BRCAX-C group compared to normal breast tissue, and higher expression of Cluster 3 miRNAs. This expression profile resembles that of BRCA1/2 tumors. In BRCAX-B group we also detect loss of expression of Cluster 2, but not of Cluster 1 or Cluster 3 compared to normal breast tissue. BRCAX-A group exhibited a similar pattern of expression to that of normal breast tissue. BRCAX-D group lacks any specific miRNA signature and in that respect it resembles sporadic breast tumors.

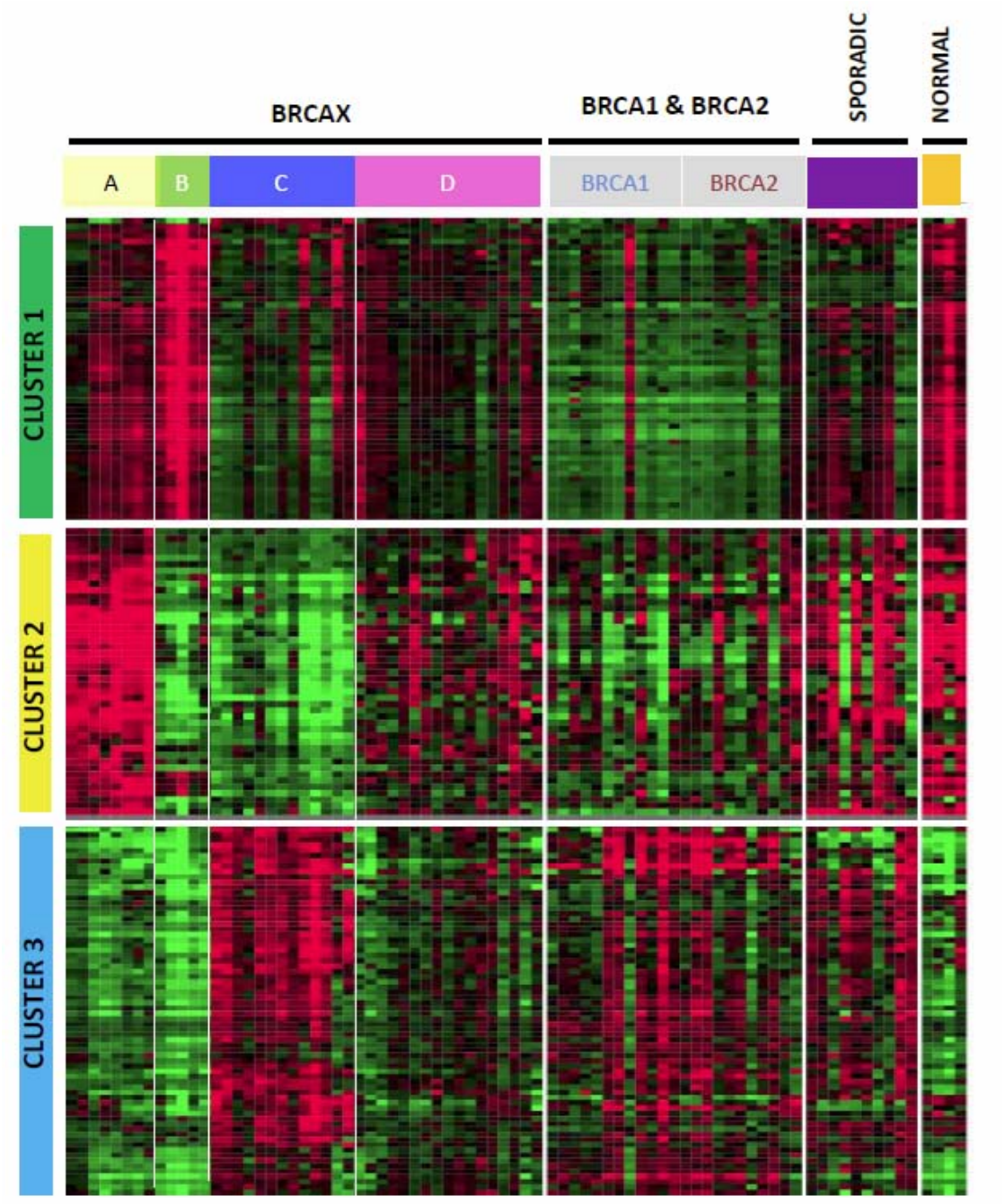


Figure 32. Supervised clustering of 43 BRCAX tumors, 23 BRCA1/2-mutated tumors, 10 sporadic breast tumors and 10 normal breast tissues over 180 miRNAs belonging to signature miRNA Cluster 1 (62 miRNAs), Cluster 2 (48 miRNAs) and Cluster 3 (70 miRNAs). Tumor samples are represented in columns and miRNA gene expression in rows in a read-black-green pseudo-color scale. Red corresponds to expression higher than media, black equal to median and green lower than median.

In order to quantify these observations we have analyzed the expression correlation of BRCAX subgroup specific miRNA signature consisting of all tree miRNA Clusters (185 miRNAs), with their expression in BRCA1/2 mutated tumor, sporadic breast tumors and normal breast tissues by linear regression using standard least-square correlation metrics (**Figure 33**). Highest correlation in miRNA expression for BRCAX-A group was observed with normal breast tissue (R^2=0.78). Expression of signature miRNAs in BRCAX-B tumors did not correlate with any of the groups compared. BRCAX-C tumors demonstrated highest correlation in miRNA expression with BRCA1/2 mutated tumors (R^2=0.0.79), while BRCAX-D tumors had highest correlation in expression to sporadic breast carcinomas (R^2=0.90).

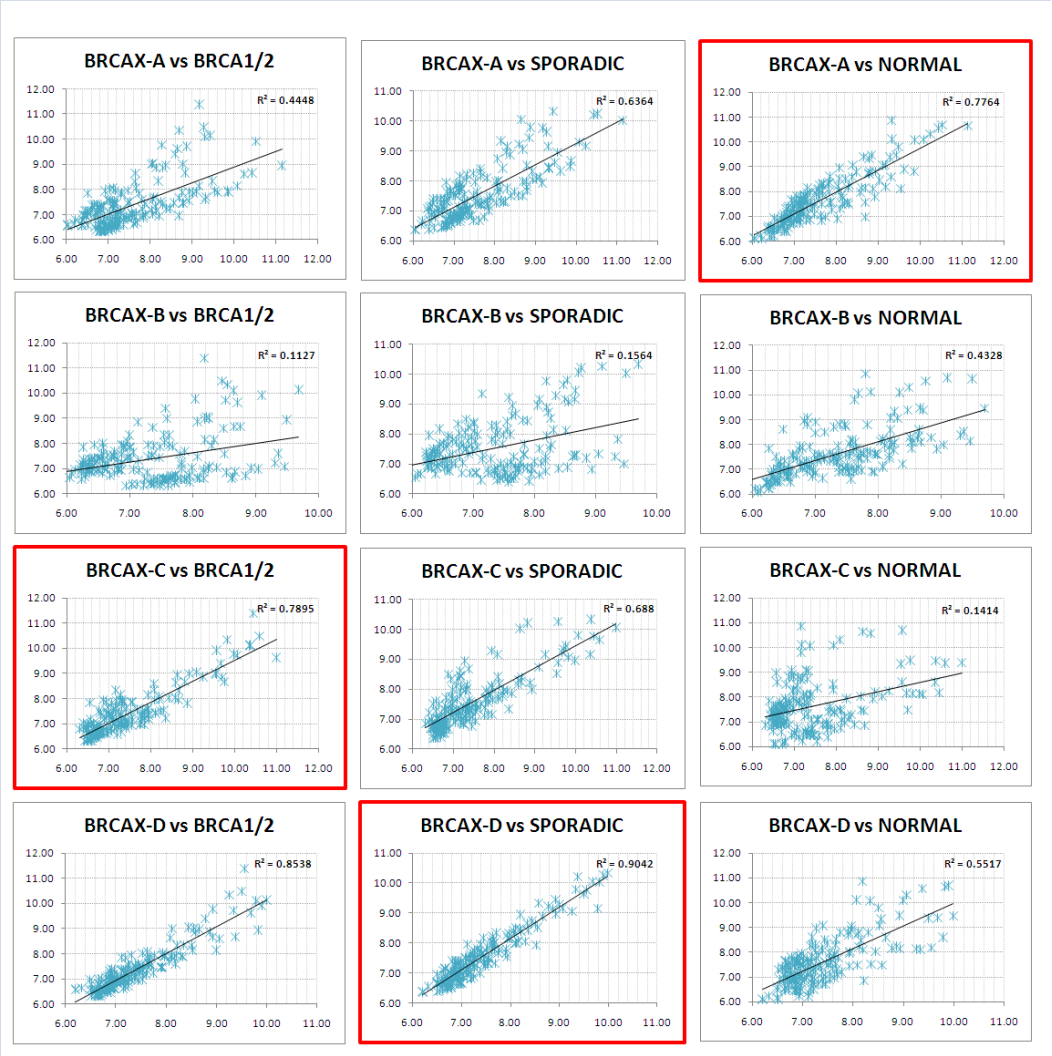


Figure 33. Plot of miRNA expression correlation for 180 miRNAs belonging to three signature clusters (Cluster 1, Cluster 2 and Cluster 3) between BRCAX subgroups (A, B, C and D), BRCA1 and BRCA2 – mutated tumors, sporadic breast tumors and normal breast tissue. Highlighted in red are most highly correlated expression profiles for each of the subgroups.

Table 8. Summary of differentially expressed miRNAs (FDR adjusted p-value <0.05) by limma t-test analysis comparing miRNA expression profiles of BRCAX subgroups to normal breast tissue

TUMOR TYPE	Up-regulated miRNAs	Down-regulated miRNAs	Total differentially expressed miRNAs
BRCAX-A	7	7	14
BRCAX-B	1	37	38
BRCAX-C	67	85	152
BRCAX-D	18	56	74

In order to quantify the level miRNA of deregulation within each of BRCAX subgroups we have performed differential expression analysis comparing each subgroup to normal breast tissue (**Supplementary table 5, 6 and 7**). Only 14 miRNAs were differentially expressed between BRCAX-A and normal breast tissue samples, while other BRCAX subgroups demonstrated much greater miRNA deregulation, with BRCAX-C having the largest number of deregulated miRNAs (**Table 8**). In addition BRCAX-C tumors had 64 miRNAs specifically deregulated in this subgroup (**Figure 23A**), while only two miRNAs, miR-143 and miR-125b, were commonly deregulated in all four groups showing low levels of expression within each subgroup (**Figure 23B**).

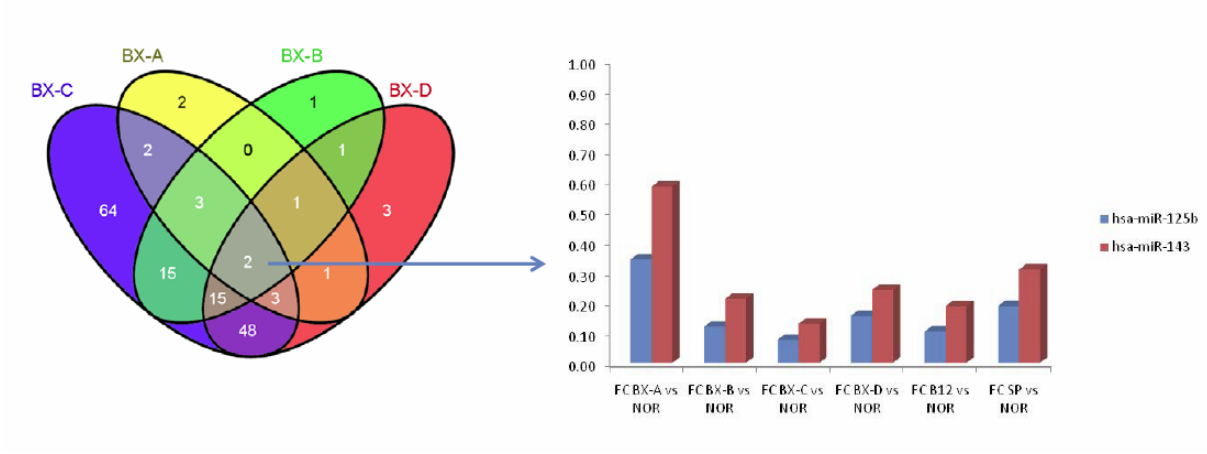


Figure 34. A) Venn diagram showing the number of common and specifically deregulated miRNAs in each subgroup **B)** Bar plot showing expression levels of commonly differentially expressed miRNAs in all BRCAX subgroups relative to normal breast tissue

3.6. Deregulated miRNA families and clusters

The identified miRNA Clusters show subtype specific pattern of expression. Both BRCAX-B and BRCAX-C subgroups are characterized by loss of expression of miRNA Cluster 2 in comparison to normal breast tissue. This miRNA cluster is composed of 48 miRNAs among which are 5 broadly conserved miRNA families, including 7 out of 9 known members of let-7 family (let-7a, let7b, let7-c, let-7d, let-7f, , let-7g, let-7i), all know miR-30 family members (miR-30a, miR-30c, miR-30d, miR-30b, miR-30e), all miR-15 family members (miR-15a, miR-16, miR-15b, miR-195), 4 out of 5 miR-8 family members (miR-200b, miR-200c, miR-200a, miR-141) and all miR-29 family members (miR-29a, miR-29b, miR-29c), in addition to miR-125b and miR-143. Interestingly, miR-8 family is upregulated in BRCAX-A tumors, and downregulated in BRCAX-C and BRCAX-B subgroup. Within miRNA Cluster 1, which was specifically downregulated in BRCAX-C tumors, we observed an enrichment of very large miRNA cluster located at 19q13.41-42 consisted of 9 primate-specific miRNAs (miR-99b*, miR-373, miR-517c, miR-519c-3p, miR-519d, miR-520d-3p, miR-520h, miR-522 and miR-526b). Members of the miRNA Cluster 3, overexpressed specifically in BRCAX-C group, included 6 out of 11 members of the primate-specific miR-506 family (miR-508-5p, miR-509-3-5p, miR-510, miR-513b, miR-509-5p, miR-513a-5p) located at a very large miRNA cluster at chromosome Xq27.3 region, and miR-154 family members (miR-381, miR-323-3p and miR-494) and miR-299-3p located at miRNA cluster at 14q32.31.

Full list of miRNAs within each Cluster, their genomic location, FDR-corrected p-values and fold change in expression comparing to normal breast tissue within each tumor group is represented in **Supplementary Tables 5, 6 and 7**.

3.7. Pathway enrichment analysis of miRNA cluster target genes

Each miRNA is predicted to regulate a large number of genes, and each gene can be a target of many different miRNAs. To determine the potential biological impact of differential expression of these miRNA clusters between BRCAX subgroups we south to identify their target genes and associated pathways. Available target prediction algorithms have high false positive rates which, given the large number of miRNAs per cluster, gave unreasonably high number of potential target genes (several thousands) hindering any pathway enrichment analysis. Therefore we have focused our analysis only on confirmed miRNA targets. For each miRNA in a cluster we have compiled a list of experimentally validated target genes from TarBase (http://diana.cslab.ece.ntua.gr/tarbase)(Vergoulis et al.) , miRTarBase (http://mirtarbase.mbc.nctu.edu.tw), miRecords (http://mirecords.biolead.org) and Ingenuity Systems Knowledge database (http://www.ingenuity.com). We performed gene-set enrichment analysis to highlight the most relevant Gene Ontology (GO) terms associated with a given gene list of mRNA targets within each miRNA cluster, using DAVID 6.7 bioinformatic algorithm (http://david.abcc.ncifcrf.gov) (Huang da et al., 2009). Top 10 statistically significant GO functional categories enriched in each dataset are represented in **Table 9**. Analysis of target genes for miRNA Cluster 2 revealed enrichment in terms related to cell death regulation, cell cycle regulation, and cell proliferation. This miRNA cluster is commonly downregulated in BRCAX-B and BRCAX-C group implying that their target genes are upregulated. Cluster 1 miRNA target genes were significantly associated in addition to regulation of cell proliferation and cell death, to regulation of cell motility and migration. This miRNA cluster is specifically lost in BRCAX-C tumors, suggesting that the mRNA targets would be upregulated. Target genes for miRNA Cluster 3, upregulated in BRCAX-C group, showed most significant association to functional terms related to regulation of cell cycle, protein kinase activity and biosynthesis of macromolecules.

Table 9. Top 10 significant GO terms enriched in miRNA Clusters

Cluster 1	GO Term	FDR
1	regulation of programmed cell death	3.8636E-12
2	regulation of cell death	4.6296E-12
3	regulation of apoptosis	6.5259E-11
4	positive regulation of cell motion	7.1054E-11
5	regulation of cell proliferation	6.8739E-10
6	regulation of cell motion	9.2683E-10
7	positive regulation of cell migration	3.6592E-09
8	positive regulation of locomotion	1.5643E-08
9	regulation of cell migration	6.5907E-08
10	regulation of locomotion	6.7346E-08

Cluster 2	GO Term	FDR
1	regulation of cell proliferation	2.3163E-23
2	positive regulation of macromolecule metabolic process	1.2576E-18
3	regulation of programmed cell death	7.4208E-17
4	regulation of cell death	9.7426E-17
5	regulation of apoptosis	3.6393E-16
6	positive regulation of developmental process	6.7366E-15
7	regulation of phosphate metabolic process	1.2121E-14
8	regulation of phosphorus metabolic process	1.2121E-14
9	positive regulation of macromolecule biosynthetic process	1.4034E-14
10	organelle lumen	1.5989E-14

Cluster 3	GO Term	FDR
1	regulation of cell cycle	1.3737E-05
2	positive regulation of macromolecule biosynthetic process	0.00039839
3	positive regulation of cellular biosynthetic process	0.00061206
4	positive regulation of biosynthetic process	0.00069981
5	positive regulation of nitrogen compound metabolic process	0.00393855
6	positive regulation of macromolecule metabolic process	0.00474381
7	regulation of protein kinase activity	0.00578571
8	transmembrane receptor protein tyrosine kinase signaling pathway	0.006535
9	regulation of kinase activity	0.00725011
10	regulation of transferase activity	0.00950461

3.8. Histopathological characteristics of BRCAX subgroups

It is well known that histology of BRCA1 and BRCA2 tumors differs from that of both BRCAX and sporadic tumors. Given that here identified BRCAX subgroups have specific miRNA signatures associated to different cellular functions, we wanted to examine whether any differences were present between subgroups on histological level. In order to test for the homogeneity of distribution of imunochistochemical markers for ER, PR, HER2, Ki-67; tumor grade and molecular subtype between subgroups of BRCAX tumors we used Fisher's exact test. No significant differences were found in the distribution of ER or PR markers, while there were significant differences in expression of HER2 and molecular subtype between different BRCAX subgroups (**Figure 4**). BRCAX-A tumors are significantly enriched for HER2 positive tumors (P=0.008) and have significantly higher proportion of Luminal B subtype (P=0.004). Of note, BRCAX-A tumors are clustering along other HER2 positive tumors in the unsupervised analysis (**Figure 19**) Furthermore, BRCAX-A tumors are significantly of lower grade tumors (only of grade 1 and grade 2) in comparison to other BRCAX subgroups. Although not reaching statistical significance, it is worth noticing that BRCAX-C tumors are consisted exclusively of grade 2 and grade 3 (**Table 10**) and are associated to lymph node positive tumors (P=0.075), while BRCAX-A tumors tend to be lymph node negative (P=0.088).

Table 10. Clinico-pathological data for BRCAX hereditary breast tumors by subtype

	BRCAX-A		**BRCAX-B**		**BRCAX-C**		**BRCAX-D**					
Total No.	**n=8**		**n=5**		**n=13**		**n=17**		**P1**	**P2**	**P3**	**P4**
	n	%	n	%	n	%	n	%				
Age at diagnosis	**7**		**5**		**12**		**16**		NS	NS	NS	NS
mean	44.71		46.60		47.25		49.81					
range	31-54		26-64		33-68		25-95					
Grade	**7**		**5**		**12**		**17**		**0.031**	NS	NS	NS
1	1	14.3%	1	20.0%	0	.0%	2	11.8%				
2	6	85.7%	2	40.0%	5	41.7%	7	41.2%				
3	0	.0%	2	40.0%	7	58.3%	8	47.1%				
Estrogen Receptor	**4**		**4**		**12**		**17**		NS	NS	NS	NS
positive	3	75.0%	2	50.0%	4	33.3%	9	52.9%				
negative	1	25.0%	2	50.0%	8	66.7%	8	47.1%				
Progesteron Receptor	**4**		**4**		**13**		**17**		NS	NS	NS	NS
positive	1	25.0%	1	25.0%	4	30.8%	8	47.1%				
negative	3	75.0%	3	75.0%	9	69.2%	9	52.9%				
HER2	**5**		**5**		**13**		**15**		**0.008**	NS	NS	NS
positive	4	80.0%	1	20.0%	1	7.7%	3	20.0%				
negative	1	20.0%	4	80.0%	12	92.3%	12	80.0%				
Subtype	**3**		**4**		**13**		**15**		**0.004**	NS	NS	NS
Luminal A	0	.0%	2	50.0%	4	30.8%	7	46.7%				
Luminal B	3	100%	0	.0%	1	7.7%	2	13.3%				
HER2	0	.0%	1	25.0%	1	7.7%	1	6.7%				
Triple Negative	0	.0%	1	25.0%	7	53.8%	5	33.3%				
Lymph Node	**6**		**3**		**12**		**14**		0.088	NS	0.075	NS
positive	1	16.7%	2	66.7%	9	75.0%	6	42.9%				
negative	5	83.3%	1	33.3%	3	25.0%	8	57.1%				

Only *P*-values<0.1 obtained by two sided Fisher's exact test are represented, with significant p-values (<0.05) in bold letters, NS=not significant. P1= *BRCAX-A* vs *rest*, P2= *BRCAX-B* vs rest, P3= *BRCAX-C* vs rest, P4=BRCAX-D vs rest. Differences in mean age at diagnos were compared by student t-test.

DISCUSSION

1. BRCA1 MEDIATED REGULATION OF MICRORNAS AND RELATED PATHWAYS

Central role of BRCA1 deregulation in the pathogenesis of both hereditary breast and ovarian cancer is well documented, and versatile BRCA1 functions have been demonstrated so far (Bertwistle and Ashworth, 1999; Starita and Parvin, 2003) including DNA damage repair/recombination, cell cycle control (Deng, 2006), chromatin remodeling and ubiquitylation (Huen et al., 2010) and regulation of both transcriptional activation and repression (Cable et al., 2003; Chapman and Verma, 1996). Previous studies using large scale genomic approaches have identified relevant genes important for tumorigenesis in relation to BRCA1 expression (Andrews et al., 2002; Furuta et al., 2006; Harkin et al., 1999; Hartman and Ford, 2002; Lamber et al., 2010; MacLachlan et al., 2000; MacLachlan et al., 2002). However, no studies so far have looked into BRCA1 mediated regulation of miRNA expression.

miRNAs play critical roles in control of various biological processes and are extensively implicated in cancer pathogenesis (Croce, 2009; Lima et al., 2011; Lu et al., 2005; Schmittgen, 2008; Thomson et al., 2006). Individual miRNAs can have multiple targets, and each mRNA may be regulated by multiple miRNAs. Still, due to the scarcity of experimentally validated miRNA target genes, much remains to be discovered about the cellular circuits controlled by miRNAs and their biological role. The combined effects of a miRNA on multiple targets emphasize the need to examine the net result of the complex modulation of multiple targets belonging to multiple pathways.

The first objective of this thesis was to identify miRNAs and miRNA-mRNA networks dependent on BRCA1 expression, by performing an integration analysis of genome-wide miRNA and gene expression data, in isogenic breast cancer cell lines differing in BRCA1 expression status. By applying functional studies we south to establish miRNA regulation of new genes and pathways that might be important in BRCA1 related breast tumors.

1.1. BRCA1-modulated miRNA and gene transcriptional profiles

Whole genome transcriptional profiling covering >45,000 mRNA sequences and global miRNA expression profiling with >800 human miRNAs was performed on a BRCA1 deficient, HCC1937 breast cancer cell line, and the isogenic HCC1937 stably expressing wt *BRCA1*. Reconstitution of BRCA1 expression in BRCA1-null cells had profound impact on gene expression, with a large number of genes being regulated representing 29% of the analyzed transcripts. These findings are in line with previously published results showing that depletion of BRCA1 induces large changes in

transcriptional profile (Horwitz et al., 2007; Horwitz et al., 2006; Lamber et al., 2010). It is known that BRCA1 can modulate gene expression by acting as a transcriptional co-factor. Still, the observed shift in gene expression could be also due to indirect effects of BRCA1, including potential gene expression regulation by miRNAs. Although BRCA1 reconstitution modulated approximately one third of genes, only 1% of analyzed miRNAs exhibited significant changes in expression, indicating more subtle effects.

Among miRNAs that were found to be upregulated upon BRCA1 reconstitution were miR-146a and miR-146b. These miRNAs were previously reported to regulate the expression of BRCA1 indicating an existence of a negative feedback loop (Shen et al., 2008). In addition, in silico predictions show a widely conserved binding site within BRCA1 3'UTR for miR-205, which was also significantly upregulated upon BRCA1 reconstitution. The mechanisms by which BRCA1 may induce the expression of miRNAs could be either through direct or indirect effects, and must be explored in further studies.

1.2. BRCA1 modulated miRNA-mRNA networks and related pathways

One of the challenges in interpretation of miRNA profiling data is the identification of miRNA target genes due to large percentage of false positive and false negative results provided by available target prediction algorithms (Alexiou et al., 2009; Maziere and Enright, 2007). Therefore, integrative studies trying to establish correlative relationships between miRNAs and mRNA expression would improve our understanding of the role of miRNAs in control of biological networks in cancer. Some studies have tried to jointly analyze miRNA expression data with its matching mRNA data to better understand the role of miRNA in breast cancer pathogenesis and progression (Blenkiron et al., 2007). In addition, integration of miRNA and mRNA expression data has been useful to establish an important role for miRNAs in regulating particular cellular processes such as proliferation, cell adhesion or immune response in primary breast cancer (Enerly et al., 2011).

We have performed integration analysis based on non-random association of miRNAs with their negatively correlated predicted target genes in an effort to identify statistically significant miRNA target genes relevant to our dataset. This approach revealed that several differentially expressed miRNAs (miR-99b, miR-146a, miR-146b) significantly associated to down-regulated mRNA targets. Gene set enrichment analysis allowed us to obtain an overview of key pathways likely to be modulated by BRCA1, among which were NF-κB, JNK and p38/MAPK pathways. Increased activation of MAPK/JNK in the BRCA1-null cell line was validated by in vitro pathway reporter assay, confirming at least in part the bioinformatic predictions.

The MAPK pathways are major signal transduction pathways that have been implicated in mammary epithelial cells and breast disease (Hori et al., 2000; Whyte et al., 2009). Increased activation of MAPK/JNK and MAPK/ERK in BRCA1-deficient cells is consistent with higher proliferation rates exhibited by these cells. NF-κB is a transcription factor that promotes the expression of genes involved in inflammatory and anti-apoptotic response (Baldwin, 1996; Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996). It has been demonstrated that NF-κB plays a critical role in development and progression of breast cancer (Madhusoodhanan et al., 2009; Zhou et al., 2008; Zhou et al., 2005). Furthermore, it has been proposed that BRCA1 acts as a co-activator of NF-κB (Benezra et al., 2003). Interestingly, it has been previously reported that miR-146a/b acts as a negative regulator of constitutive NF-κB activity in breast cancer setting (Bhaumik et al., 2008). In addition, NF-κB dependent induction of miR-146a/b has been established, that along with miR-146 negative regulation of *IRAK1* and *TRAF6*, constitutes a negative-feedback loop that controls the activity of NF-κB pathway (Taganov et al., 2006).

1.3. NF-κB pathway activity is modulated by miR-146a/b

In support of the aforementioned findings, we have demonstrated that miR-146a/b along with newly identified miR-99b and miR-205 can modulate NF-κB pathway activation, while MAPK/ERK can be modulated only by miR-146a. On the other hand, individually none of these miRNAs were sufficient to modulate MAPK/JNK pathway whose activity was increased in BRCA1-null cells. This result could be explained by diverse and/or contrasting functions of many target genes for each individual miRNA, emphasizing the notion that different combinations of deregulated miRNAs could have very different biological outputs.

Consistent with the bioinformatic predictions, we have shown that TRAF2 had higher expression in BRCA1-null cells. Moreover, overexpression of genes related to immune response, including *TRAF2* gene, is one of the intrinsic characteristics of in ER-negative BRCA1-mutated tumors (Fernandez-Ramires et al., 2009). Numerous studies have implicated TRAF2 as a critical mediator of NF-κB (Arch and Thompson, 1998; Duckett et al., 1997; Hsu et al., 1996; Reinhard et al., 1997; Rothe et al., 1995; Takeuchi et al., 1996), JNK and p38 activation (Liu et al., 1996; Natoli et al., 1997; Reinhard et al., 1997) and it has been reported that overexpressed native TRAF2 can activate JNK, p38 and NF-κB in the absence of extracellular stimuli (Cao et al., 1996; Liu et al., 1996; Rothe et al., 1995; Song et al., 1997). Up-regulation of TRAF2 expression in BRCA1-deficient cells due to release of negative regulation by miR-146a/b, miR-99 and miR-205 could contribute to NF-κB and MAPK pathway activation. Significantly, further validation of microarray results in the cell model, showing the down-regulation of miRNAs in HCC1937 BRCA1-null cells, came from our finding that three of

these miRNAs, miR-99a, miR-146b and miR-205, were also down-regulated in BRCA1-mutated primary breast tumors.

Figure 35. miRNA-gene network of NFkB pathway regulation

2. MIRNA DEREGULATION IN HEREDITARY BREAST CANCER

miRNA profiling studies in various tumor types have demonstrated a widespread miRNA deregulation, providing for new insights in tumor biology and opening new avenues for development of novel targeted therapies in cancer (Lujambio and Lowe, 2012). In the second study we have looked into the miRNA molecular profiles altered in hereditary breast tumors in comparison to normal breast tissue. The normal breast tissue series was obtained from healthy individuals, both from *BRCA1/2*-mutation carriers belonging to high risk families, and from mutation-free individuals undergoing breast reduction surgery. This has enabled us to pinpoint miRNAs important for tumorigenesis, by removing the bias induced through effects of BRCA1/2 mutation on miRNA expression. We have defined down-regulation for 17 miRNAs and up-regulation of miR-21 and miR-300 in hereditary breast tumors in comparison to normal breast tissue.

It was expected that comparing tumor samples with normal tissue, characterized by largely quiescent cells, would uncover miRNAs that may be mainly involved in proliferation. Expression analysis of the 19 differentially expressed miRNAs in HMEC cells, which represent normal proliferating cells, have allowed finding some miRNAs overexpressed in normal tissues that were not overexpressed in normal HMEC cells, such as miR-99a, miR-101 or miR-145, which could have a role in the control of proliferation processes in general. Other miRNAs expressed by both HMEC and normal breast and down-regulated in tumors tissue may be involved in other signaling pathways related to tumoral behavior.

2.1. Commonly deregulated miRNAs in hereditary and sporadic breast carcinomas

A high proportion of deregulated miRNAs in hereditary tumors were commonly found in previous studies with sporadic breast tumors, suggesting that there are miRNAs that likely regulate important oncogenes and tumor suppressor genes involved in both hereditary and sporadic tumor progression, irrespective of their genetic background or molecular subtype (Iorio et al., 2005; Volinia et al., 2006). These included miR-21, let-7a, miR-125b, miR-10b, miR-100, miR-101, miR-143, miR-145, miR-205 and miR-210. We shall discuss functional roles for some of them more in detail.

The let-7 family of 12 miRNAs is often cited as the archetypal tumor- suppressing miRNA. Let-7 is essential for cell type determination during embryogenesis in C. elegans and in the adult human (Reinhart et al., 2000). Its expression is highest in terminally differentiated epithelial tissues. Let-7 expression is lost at an early stage in breast cancer progression (Sempere et al., 2007), and continued

expression is associated with low-grade, ER-positive, luminal A tumours (Blenkiron et al., 2007). Let-7 is downregulated in subpopulations of murine breast cells with stem-like properties (Ibarra et al., 2007), and during epithelial to mesenchymal transition (EMT) (Dangi-Garimella et al., 2009). Several functional targets of let-7 have been identified, including the classical proto-oncogene RAS (Johnson et al., 2005), and the oncofoetal proteins HMGA2 (Mayr et al., 2007) and IMP-1 (Boyerinas et al., 2008). The manipulation of let-7 levels in breast cancer cell lines affects proliferation and metastasis, and both H-RAS and HMGA2 are involved in these responses (Yu et al., 2007).

miR-21 was one of the first oncogenic miRs to be characterized, being upregulated in numerous tumors, including breast cancer (Iorio et al., 2005). Overall, miR-21 expression in breast tumors correlates with advanced stage, and metastasis, and with poor survival independently of grade and stage (Huang et al., 2009; Yan et al., 2008). Knockdown of miR-21 inhibits MCF7-derived tumor growth in xenograft models via reduced proliferation and increased apoptosis (Si et al., 2007). Validated miR-21 targets include tropomyosin 1 (TPM1), which suppresses anchorage-independent growth in vitro (Zhu et al., 2007), programmed cell death 4 (PDCD4), (Lu et al., 2008; Frankel et al., 2008), and maspin, a tumor suppressing serpin protein (Zhu et al., 2008). miR-21 also targets the tumor suppressor PTEN in hepatocellular carcinoma, and there is a correlation between miR-21 expression and loss of PTEN expression in breast cancer (Huang et al., 2009).

Similarly, miR-125b, which was identified early in the development of the field as being downregulated in breast tumors (Iorio et al., 2005), is located at chromosome 11q23-24, one of the regions most frequently deleted in breast, ovarian, and lung tumors (Negrini et al., 1995). miR-125 suppresses cell growth and favors apoptosis in breast cancer cell lines via targeting of HuR (Guo et al., 2009), itself an independent prognostic marker in breast cancer (Heinonen et al., 2005). It also suppresses the growth of HER2-dependent SKBR3 cells when artificially over-expressed, via the targeting of HER2 and HER3 (Scott et al., 2007). Finally, miR-125b has also been shown to target the tumor-associated protein CYP24, which metabolizes antiproliferative calcitriol (Komagata et al., 2009

In the normal breast expression of miR-205 is restricted to basal epithelium (Sempere et al., 2007). Its expression is generally lost early in breast cancer progression, but persistent (albeit lowered) expression is linked to the presence of basal immunohistochemical markers. Within ER/PR/HER2-negative tumors, loss of miR-205 is a poor prognostic indicator (Sempere et al., 2007). miR-205 is downregulated in EMT, along with the miR-200 family, and like miR-200 targets both ZEB1 and ZEB2 (Gregory et al., 2008). It also targets HER3, which co-operates in the mitogenic HER2 pathway (Iorio et al., 2009), and miR-205 overexpression both reduces proliferation in cell culture, and increases sensitivity to tyrosine kinase inhibitors. Another study showed that miR-205

suppresses lung metastasis in an animal model, and implicates HER3 and VEGF-A (Wu et al., 2009a,b).

miRNAs can function both as oncogenes or tumor suppressor via negative inhibition of tumor suppressor genes or oncogenes in pathways that control cellular differentiation, proliferation or apoptosis. All these findings overwhelmingly implicate the let-7 family, miR-205, miR-125b as a tumor-suppressive miRNAs in breast setting, while in general, miR-21 up-regulation is considered to be a common feature of proliferative tissues.

2.2. Analysis of miRNA- associated pathways

Moreover, the pathway enrichment analysis suggested that miRNAs co-expressed in our series, seem to collectively target a broad range of signaling pathways related to proliferation and cell migration/motility. Altered cell signaling has long been recognized as a mechanism employed by cells in the development and progression of cancer (Hanahan and Weinberg, 2000).

Importantly, 20 genes within MAPK signaling pathway were significantly associated with our set of deregulated miRNAs, suggesting that the inhibition of these miRNAs would result in a concomitant activation of MAPK signaling. In breast cancer, MAPKs play a key role in transducing growth signals from the extracellular environment (Dunn et al., 2005; Haagenson and Wu, 2010). The activation of the KRAS/MAPK pathway generates a plethora of responses in breast cancer tumors and cell lines, affecting cell growth, proliferation, differentiation and transformation (Atanaskova et al., 2002). In breast cancer development, up-regulation of the KRAS/MAPK signaling can occur through multiple facets, and it has been shown to be increased in many breast cancer samples either by over-expression of growth-factor-receptor tyrosine kinases primarily HER2/ErbB-2, EGFR, and IGFR or by activating mutations (Eckert et al., 2004; Lo et al., 2006; McCubrey et al., 2007; Salh et al., 1999). Although KRAS is frequently mutated in human cancers including pancreatic, colorectal and lung cancers, KRAS mutations are very rare in breast cancer (Clark and Der, 1995; Koffa et al., 1994). However wild-type KRAS is significantly activated in breast cancers that over-express EGFR and HER2 (von Lintig et al., 2000). Additionally many investigators have reported over-expression of the KRAS-encoded p21 proteins in breast malignancies in comparison to normal breast tissue (Agnantis et al., 1994; Spandidos, 1987) although the role of this over-expression in breast carcinogenesis has not been determined.

2.3. KRAS oncogene is a target of miR-30c in breast cancer

Interestingly, KRAS was found to be a target of multiple miRNAs found to be down-regulated in breast tumors. The let-7 family of miRNAs has been shown to regulate multiple oncogenes, including KRAS and c-MYC (Johnson et al., 2005), and miR-143/145 are involved in feed-forward mechanism that potentiates RAS signaling through down-regulation of KRAS and RAS-responsive element-binding protein (RREB1), which represses the miR-143/145 promoter (Kent et al., 2010). Here we have identified a novel broadly conserved miRNA, miR-30c, as a direct negative regulator of KRAS expression. Interestingly, miR-30 and let-7 were reported to be markedly reduced in breast tumor-initiating cells and contribute to their self-renewal capacity and undifferentiated state, and ectopic expression of these miRNAs in breast tumor-initiating cell xenografts decreases their tumorigenic and metastatic potential (Yu et al., 2010a). Furthermore, it has been shown recently that higher expression of miR-30c was significantly associated to benefit of tamoxifen treatment and with longer progression-free survival (Rodriguez-Gonzalez et al., 2011). Altogether, decreased expression of these miRNAs may release the negative regulation of KRAS. Interestingly, our results showed that at least three KRAS regulating miRNAs (miR-30c, miR-143, and miR-145) had significantly reduced expression in hereditary tumors implying that these miRNAs may act together in the regulation of KRAS oncogene.

Signal transduction pathways integrate signals from extracellular stimuli including mitogens, growth factors, hormones and environmental stresses- signals required for tumorigenesis. miRNA deregulation results in the complex modulation of multiple targets belonging to multiple pathways. Commonly deregulated miRNAs in both hereditary and sporadic breast cancer suggest that commonly altered pathways could be important for tumor progression. Here, we have demonstrated that KRAS inhibition through direct regulation by miR-30c leads to reduced proliferation in breast cancer cells. Similarly, other studies have identified KRAS as a target of several miRNAs down-regulated in tumors (let-7, miR-145 and miR-143), that also have an effect on cancer cell proliferation and tumor invasiveness (Chen et al., 2009; Johnson et al., 2005; Yu et al., 2010b). Therefore, coordinated down-regulation of miRNAs found in breast tumors would be not only affecting KRAS oncogene expression but also may be targeting other genes of the KRAS/MAPK signaling pathway to cooperatively activate tumorigenic downstream signals.

3. MIRNA MOLECULAR SIGNATURES IN HEREDITARY BREAST TUMORS

Breast cancer is a heterogeneous disease, and in the past two decades great advances have been made by use of mRNA expression profiling for stratification of sporadic breast tumors into subgroups with distinctive clinico-pathological features, prognosis and responses to therapy (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003). However, molecular characteristics of hereditary breast tumors, especially those arising in non-*BRCA1/2* mutation carriers are still largely obscure. miRNA expression profiling has proven to be a useful tool for molecular classification of human cancer (Lu et al., 2005; Lujambio and Lowe, 2012). Therefore, the third objective of this thesis was to explore the molecular phenotype of BRCA1, BRCA2 and BRCAX hereditary breast tumors based on their intrinsic molecular characteristics by miRNA expression profiling.

Studies of transcriptional profiles in hereditary breast tumors were limited largely by the requirement of the fresh-frozen tumor material, resulting in only few publications describing mRNA expression profiles in these tumors. On the other hand, miRNA expression profiling has proven to be a very useful tool for cancer classification, and, due to the remarkable stability of miRNAs in formalin fixed paraffin embedded tissue samples (Hasemeier et al., 2008), it has opened up an opportunity to use large collections of archived FFPE samples. In this study, we have analyzed by miRNA expression profiling a cohort of 66 hereditary breast tumors (13 BRCA1, 10 BRCA2 and 43 BRCAX), 10 sporadic breast carcinomas and 6 normal breast tissues from healthy individuals with no personal of family history of breast cancer. Since contamination with normal breast tissue and stroma is one of the major issues in gene expression profiling studies, all samples analyzed in our series were macrodissected and isolated tumoral area was used for the total RNA extraction. All patents' samples originate from Spanish hospitals, reducing the heterogeneity due to potential population-specific founder mutation effects.

3.1. miRNA expression profiles differ between breast tissue types.

The unsupervised clustering analysis of the miRNA expression data demonstrated clear separation between normal breast tissue, sporadic carcinomas, BRCA1 and BRCA2 tumors, and BRCAX tumors, supporting the presence of unique molecular differences between these groups. BRCAX tumors show large heterogeneity in their miRNA expression profiles, but overall they differ from both BRCA1/2 and sporadic tumors.

In line with our results, a recent study of a large series of hereditary breast samples and sporadic carcinomas (58 BRCAX, 28 BRCA1, 28 BRCA2, 49 sporadic) by aCGH, demonstrated that BRCAX tumors constitute a heterogeneous class, and are distinct from both sporadic and BRCA1/2 tumors (Didraga et al., 2011). In addition, the analysis of methylation profiles in a series of 33 hereditary breast cancers, also demonstrated that methylation profiles for hereditary breast cancers are defined by mutation status and are distinct from the intrinsic subtype determined by gene expression profiling in the same sample series (Flanagan et al., 2010)

Interestingly, these findings are in contrast to results obtained by gene expression profiling and IHC-based profiling of hereditary breast tumors (Bane et al., 2009; Melchor et al., 2008; Waddell et al., 2010). Gene expression profiling of 75 hereditary breast tumors revealed that majority of BRCA2 and BRCAX tumors were more similar to each other than to BRCA1 tumors. However this clustering was mainly driven by molecular subtypes given the enrichment of basal-like subtype within BRCA1 tumors (Waddell et al., 2010). Here we have shown that clustering of hereditary tumors based on their miRNA expression profiles did not correlate with the IHC-based subtypes. These results are in concordance with previous findings on miRNA and gene expression profiling data in sporadic breast carcinomas (Rothe et al.), indicating that the miRNAs-based clustering of hereditary breast tumors is not driven by molecular subtypes and may carry additional information on these tumors.

However, clustering analysis of miRNA expression data showed bi-partitioning that correlated with tumor grade, dividing the samples into a “low-moderate” grade cluster containing mainly BRCAX tumors and a “high grade” cluster consisted of two subgroups, one made of exclusively BRCA tumors and other mainly with BRCAX tumors and two BRCA1 samples. The separation into a “high grade” and “low grade” clusters was also reported in study of BRCAX tumors by IHC Honrado et al. implying that tumor differentiation level is driving the main stratification (Honrado et al., 2007).

3.2. miRNA signature of “BRCAness”

In the unsupervised clustering analysis it was shown that BRCA1 and BRCA2 tumors have very similar miRNA expression profiles, which was further supported by the lack of significantly differentially expressed miRNAs between these two groups. Furthermore, a large number of miRNAs (152) demonstrated significant differential expression between BRCA tumors and other mutation-negative breast tumors constituting a signature of “BRCAness”.

Of note, in a study of miRNA expression profiles in high grade ovarian serous carcinomas in relation to *BRCA1/2* mutation status, no differentially expressed miRNAs were found between BRCA1 and BRCA2 tumors (Lee et al., 2009). However, the homogeneity of BRCA1/2 group determined by their miRNA expression profiles is in contrast to studies of gene expression in

hereditary tumors (Bane et al., 2009; Hedenfalk et al., 2001; Waddell et al., 2010). Hedenfalk et al. identified 173 differentially expressed genes between the two tumor types and a more recent study comparing expression in 19 BRCA1 and 30 BRCA2 tumors identified 393 genes (Hedenfalk et al., 2001; Waddell et al., 2010). It is well established that BRCA1-associated tumors differ from BRCA2 in terms of their morphological, immunohistochemical and molecular characteristics (Honrado et al., 2006; Honrado et al., 2005; Lacroix and Leclercq, 2005; Mavaddat et al., 2011; Tan et al., 2008; Tischkowitz and Foulkes, 2006; Turner and Reis-Filho, 2006; Vargas et al., 2011). Interestingly, one of the two miRNAs downregulated in BRCA1 tumors in comparison to BRCA2, although not significant after multiple corrections testing, miR-7, is known to regulate EGFR gene expression. EGFR mutations are a frequent even in BRCA1 tumors, but not in BRCA2, and downregulation of EGFR repressor, miR-7, could result in its overexpression.

Cancers that develop in patients with *BRCA* germline mutations are characterized by distinctive morphology and clinical behavior. Both genes are involved in a common pathway of genome protection by homologous recombination of double-stranded DNA brakes (Roy et al., 2012). However, the two proteins work at different stages in the DNA damage response and DNA repair. The links between the two proteins are not well understood but they must exist to explain the marked similarity of human cancer susceptibility that arises with germline mutations in these genes. High similarity in miRNA expression profiles of BRCA1 and BRCA2 tumors possibly reflects the overlapping functions of these two genes in the DNA double strand break repair pathway, implying that miRNA expression profile is in function of a biological pathway deregulation rather than of a specific gene functions.

The high homogeneity of BRCA1 and BRCA2 tumors and a large number of differentially expressed miRNAs in these tumor group versus BRCAX and sporadic breast carcinomas could allow for the development of biomarkers of diagnosis that would potentially discriminate between mutation positive and negative tumors and facilitate identification of individuals at risk of being mutation carriers that would benefit from genetic testing and would be amenable to DNA damage-inducing therapy, such as platinum and PARP inhibitors. Identifying *BRCA1/2* specific biomarkers would be of great clinical value for pre-selection of candidate patients for *BRCA1/2* mutation screening, especially among patients with lack of affirmative family history due to small pedigree or paternal transmission.

4. MIRNA-BASED MOLECULAR CLASSIFICATION OF BRCAX TUMORS

The underlying biology of *BRCA1/2*-negative hereditary breast cancer is still largely unknown despite intensive research in past two decades. The notorious heterogeneity of BRCAX tumors has been demonstrated using various technological platforms including gene expression profiling, comparative genomic hybridization (CGH), immunohistochemistry, LOH and methylation studies (Honrado et al., 2007). Understanding the biology behind BRCAX tumor heterogeneity could allow for guiding optimal therapeutic and preventive options, and may help stratify BRCAX families for linkage analysis. Here we have examined weather the heterogeneous genetic background of BRCAX tumors could be resolved using miRNA expression profiling.

4.1. miRNA expression signatures define BRCAX subgroups

Unsupervised clustering of BRCAX tumors based on their miRNA expression demonstrated once again the heterogeneous nature of these tumors, with several apparent subgroups. Class discovery algorithm confirmed the existence of 4 different subgroups of tumors, characterized by a specific miRNA signature. Three miRNA clusters based on their co-expression were defined. BRCAX-A tumors showed overall similar levels of miRNA expression to normal breast tissue. BRCAX-B tumors on the other hand were differentiated from normal tissue only by the down-regulation of Cluster 2 miRNAs. The miRNA expression profile of BRCAX-C tumor group demonstrated inverse expression levels to that of normal breast tissue. It was characterized by downregulation of Cluster 1 and Cluster 2 miRNAs, and overexpression of Cluster 3 miRNAs. However, BRCAX-D tumors exhibited very heterogeneous profiles. Only two miRNAs, miR-125b and miR-143, were commonly deregulated in all four subgroups in comparison to normal breast tissue. Interestingly, miR-125b, which is downregulated in breast cancer, is located at chromosome 11q23-24, one of the regions most frequently deleted in breast, ovarian, and lung tumors (Negrini et al., 1995). Functional roles of these tumor suppressor miRNAs have been previously discussed.

Cluster 1, specifically downregulated in BRCAX-C tumors, was enriched for a large number of primate-specific miRNAs localized at chromosome 19q13.41-42 (Zhang et al., 2008). Interestingly, this region is genetically altered in a variety of tumors featuring deletions, amplifications and translocations(Forzano et al., 2012; Toledo et al., 2010), and C19MC miRNA cluster has been extensively implicated in cancer and other diseases (Pfister et al., 2009; Rippe et al., 2010; Vaira et al., 2012). Bioinformatic analysis of Cluster 1 miRNA target genes related their function to positive regulation of cell migration and motility, in addition to regulation of cell proliferation and apoptosis.

Cluster 2, downregulated in BRCAX-B and BRCAX-C tumors, contained well known tumor suppressor miRNAs implicated in breast pathogenesis of both sporadic and hereditary cancer (Le Quesne and Caldas, 2010; Tanic et al., 2012), including all members of let-7 and miR-30 family, that are controlling cell proliferation and differentiation in breast setting, as discussed in previous chapters. Other tumor suppressor miRNAs in this cluster included commonly altered miR-125b, miR-143. Loss of tumor suppressor miRNAs would lead to concomitant increase in proliferation rates and de-differentiation, hallmarks of cancer. Interestingly, the miR-8 family members (miR-141/200a/200b/200c) were more than 2-fold upregulated in BRCAX-A tumors, while being downregulated in BRCAX-B and BRCAX-C subgroups. This miRNA family acts as suppressors of epithelial to mesenchymal transition (EMT) (Burk et al., 2008), and is also implicated in the maintenance of stem cell phenotype, and downregulated in mammary progenitor cells (Shimono et al., 2009). As expected, genes related to miRNA Cluster 2 were associated to pathways regulating cell death, cell proliferation, developmental processes and macromolecule metabolism.

Cluster 3 miRNAs were specifically overexpressed in BRCAX-C tumors and contained a very large primate-specific miRNA cluster located at chromosome Xq27.3 (Zhang et al., 2008). Notewotrhy, this region was linked to testicular germ cell tumors (Rapley, 200, Nat Genet), hereditary prostate cancer (Peters, Hum Hered, 2001; Lange ClinCancRes, 1999) and miRNAs in the cluster were implicated in clear cell renal crrcinoma (Zhou et al., 2010). Biological functions associated to target genes of Cluster 3 miRNAs were regulation of cell cycle, protein kinase activity and biosynthesis of macromolecules.

4.2. miRNA profiles may indicate differences in tumor etiology

It is known that miRNA expression is highly specific for the tissue of origin and developmental stage (Landgraf et al., 2007; Niwa and Slack, 2007). In effect, miRNA profiling is emerging as a powerful diagnostic tool to characterize features of different tumor types and has been particularly useful in breast cancer as miRNA signatures can unequivocally distinguish normal ad malignant breast tissue. Furthermore, we have shown that BRCA1 and BRCA2 germline mutations involved in the specific pathway give rise to a very characteristic miRNA signature. Therefore, we can hypothesize that deregulation of specific miRNA could indicate different pathways of tumor evolution in each of the BRCAX subgroups that may harbor predisposing mutations in the same pathway.

Deregulation of two different processes is converging in of the same macro-phenotype, the occurrence breast cancer. Genomic instability driven by inactivation of functional BRCA1, BRCA2 and other “care-taker” proteins important for DNA damage repair are common cause of highly penetrant hereditary breast cancer syndromes. It is currently believed that accumulation of genetic and

genomic alterations over the years, due to faulty DNA repair mechanisms is causing the malignant phenotype. On the other hand, over-activation of signaling pathways regulating cellular growth and proliferation is a very frequent event in breast cancer. Deregulation of MAPK pathway via HER2 receptor overexpression is a well known driver of breast tumorigenesis in approximately 20% of breast tumors. Another frequent event is deregulation of PI3K/AKT signaling evidenced by the highest frequency of somatic mutations in breast tumors in PIK3CA gene (26% of tumors) according to data from the Catalogue of The Somatic Mutations in Cancer (COSMIC, http://www.sanger.ac.uk/genetics/CGP/cosmic/).

Lack of association of hormone receptor status with a specific tumor subgroup excludes the possibility that miRNA signatures characterizing these tumor subgroups are associated with endocrine response to estrogen or progesterone. However, other underlying genetic alterations or external signaling could be reflected in the observed miRNA profiles. BRCAX-A tumors were the only subgroup significantly associated with specific histopathological features, specifically to HER2 overexpression. Although HER2 overexpression is associated to poor prognosis, these tumors were significantly of moderate grade ($p=0.03$) and lymph node negative ($p=0.08$) both of which are clinical parameters for good prognosis. miRNA expression profile in these tumors closely resembles that of normal breast tissue, with only few differentially expressed genes unlike massive miRNA deregulation observed in other tumor subgroups, implying that in these tumors overactive HER2 signaling is sufficient for tumorigenesis process. The BRCAX-B subgroup of tumors was not significantly different in terms of histopathological characteristics from the rest of tumors. In fact these tumors are characterized by the downregulation of tumor suppressor miRNAs, important for pathogenesis of both hereditary and sporadic tumors. On the other hand, BRCAX-C tumors in addition to downregulation of Cluster 2 tumor suppressor miRNAs had subtype-specific aberrantly expressed miRNAs. These tumors were consisted only of high and moderate grade tumors, although not statistically significant, and were enriched for lymph node positive tumors ($p=0.075$), both characteristics being related to poor prognosis. Given the high similarity in miRNAs expression profiles between BRCAX-C tumors and BRCA1/2 mutated ones, this subgroup of tumors would represent good candidates for resequencing studies focused on the DNA-damage repair pathway.

It is very likely that the group of BRCAX cases is attributable to multiple genetic defects. It is not known how many additional breast cancer susceptibility genes exist, nor how many may fall into the class of rare high-risk (BRCA-like) or common low susceptibility alleles. However, given that linkage studies have failed to identify new susceptibility genes, it is to assume that even if more highly penetrant genes exist, they will not explain more than 20% of remaining families. The power of linkage analysis would increase dramatically if we were able to select a sub-group of patients that would increase the proportion of this potential high-risk gene to over 50%. Differences in tumor

etiology reflected in the miRNA expression profile, evidenced by specific signature associated to BRCA1 and BRCA2 tumors, could be exploited patients stratification for linkage analysis or allow for studies of interaction of low-susceptibility alleles in homogenous set of tumors.

5. FINAL REMARKS

Although miRNA deregulation is well established phenomenon in cancer, and the downstream effects of miRNAs are an intensive object of study, there are surprisingly few studies focused on the underlying causes of miRNA deregulation. The genomic abnormalities found to influence the activity of miRNAs are the same as those previously described for protein-coding genes, such as chromosomal rearrangements, genomic amplifications or deletions, and somatic or germline mutations (Calin et al., 2004a; Calin et al., 2004b). Weather massive miRNA deregulation is the cause or the consequence of tumorigenesis is still an open question. Recurrent deregulation of specific miRNAs in breast cancer located across different regions of the genome implies that either there is a common factor regulating their expression, or that deregulation of specific miRNAs through random genetic or epigenetic alterations arising over the years is a necessary event for the breast tumorigenesis. This question will undoubtedly be a matter intense investigation in years to come and will hopefully shed some light on the functional role of miRNA in cancer pathogenesis.

CONCLUSIONS

1. Reconstitution of wild type BRCA1 expression in HCC1937 cell lines induces large changes in cellular transcriptional profile and only subtly modulates miRNA expression. Integration of the global miRNA and mRNA profiles allowed us to define genes and pathways controlled by differentially expressed miRNA upon BRCA1 re-expression. MAPK and NF-κB pathways were differentially activated between BRCA1-profficient and deficient HCC1937 cell lines

2. We have identified TRAF2, a well known mediator of both NF-κB and MAPK pathway activation, as a novel common target gene for miR-146, miR-99 and miR-205, upregulated upon BRCA1 reconstitution. We demonstrated that these miRNAs individually are sufficient to modulate NF-κB pathway activity in breast cancer cells. Furthermore, we observed that miR-146, miR-99 and miR-205 were also downregulated in primary BRCA1 tumors in comparison to normal breast tissue.

3. We have analyzed, for the first time, miRNA expression profiles in hereditary breast tumors and defined a set of 19 miRNAs deregulated in hereditary breast tumors, many of them commonly deregulated in sporadic breast cancer.

4. Gene set enrichment analysis indicated that this set of co-expressed miRNAs regulates pathways related to cell proliferation and cell migration. We identified KRAS a novel target gene of miR-30c in breast cancer cells. Re-expression of miR-30c in MDA MB 436 breast cancer cells inhibited cell proliferation.

5. Classification of breast tissues based on their miRNA expression profiles demonstrated clear separation of BRCA1/2 tumors, BRCAX tumors, sporadic carcinomas and normal breast tissue.

6. Hereditary breast tumors from BRCA1/2 mutation carriers represented a highly homogenous group, with no differentially expressed miRNAs between BRCA1 and BRCA2 tumors. A large number of miRNAs was differentially expressed between BRCA1/2 mutation positive and mutation negative breast tumors that can serve as a starting point for the development of biomarkers of diagnosis for triage of individuals at risk of being mutation carriers that would benefit from genetic testing and emerging therapies.

7. The group of hereditary BRCAX tumors can be subclassified into 4 homogenous groups, BRCAX-A, BRCAX-B, BRCAX-C and BRCAX-D determined by specific miRNA expression signatures. BRCAX-A tumors demonstrated high correlation in miRNA expression profile with normal breast tissue, were enriched for Luminal B subtype (HER2+,ER/PR+) and consisted of only grade 1 and 2 tumors. BRCAX-B tumors represented a homogenous group characterized by loss of expression of “tumor suppressor” miRNA Cluster 2. BRCAX-C tumors showed similar miRNA expression profile to BRCA1/2 mutated tumors and clustered in the same branch; were characterized by loss of “tumor suppressor” miRNA cluster, loss of miRNA Cluster 3 related to migratory functions and overexpression of miRNA Cluster 3 associated to metabolism of macromolecules. BRCAX-D tumors were lacking a specific miRNA signature and were dispersed across the cluster. Their miRNA expression profile resembles that of sporadic breast tumors.

CONCLUSIONES

1. La recuperación de la expresión del gen BRCA1 wt en HCC1937 líneas celulares provoca grandes cambios en el perfil transcripcional y sólo modula sutilmente la expresión de los miRNAs. La integración del perfil global de miRNA y de mRNA nos permitió definir los genes y las vías controladas por miRNA expresados diferencialmente tras la re-expresión de BRCA1. Existen diferencias en la activaci'on de las v'ias MAPK y NF-kB entre Las líneas celulares isogenicas HCC1937 que expresan BRCA1 y que carecen de esta expresión.

2. Hemos identificado TRAF2, un mediador del activación de las vías NF-kB y MAPK, como un nuevo gen diana común para los miR-146, miR-99 y miR-205 que están sobre-expresados en la línea HCC1937/ BRCA1 wt. Hemos demostrado que cada miRNA individualmente es suficiente para modular la actividad de la vía NF-kB en células de cáncer de mama. Asimismo, se observó que el miR-146, miR-99 y miR-205 también se expresan menos en los tumores BRCA1 en comparación con el tejido normal mamario.

3. Hemos analizado, por primera vez, los perfiles de expresión de los miRNAs en los tumores de mama hereditarios pudiendo definir así un conjunto de 19 miRNAs que están desregulados en estos tumores de los cuales la gran mayoría son comunes a los miRNAs desregulados en cáncer de mama esporádico.

4. Análisis de enriquecimiento de las vías de señalización en el conjunto de genes asociados a los miRNAs co-expresadas nos indicó que estos miRNAs regulan indirectamente las vías relacionadas con la proliferación celular y la migración celular. Hemos identificado el oncogén KRAS como nueva diana del miR-30c en las células de cáncer de mama. La re-expresión del miR-30c en las células de cáncer de mama MDA MB 436 inhibe la proliferación celular.

5. Clasificación de los tejidos mamarios en función de sus perfiles de expresión de los miRNAs ha demostrado una clara separación entre los tumores BRCA1 / 2, tumores BRCAX, carcinomas esporádicos y el tejido mamario normal.

6. Los tumores de mama hereditarios de los portadores de mutaciones BRCA1 / 2 representan un grupo muy homogéneo, sin ningún miRNA expresados diferencialmente entre los tumores BRCA1 y BRCA2. Un gran número de los miRNAs mostraron una expresión diferencial entre tumores positivos y negativas para la mutación BRCA1 /2, que podrían servir como arranque para el desarrollo de los biomarcadores de diagnóstico para la clasificación de los individuos en riesgo de ser portadores de la mutación que se beneficiarían de las pruebas genéticas y terapias emergentes.

7. Entre el grupo de los tumores hereditarios BRCAX se pueden distingir 4 grupos homogéneos, BRCAX-A, BRCAX B, BRCAX-C y BRCAX-D determinados por las específicas firmas de expresión de los miRNAs. Los tumores BRCAX-A han demostrado una alta correlación en el perfil de expresión de los miRNAs con el tejido mamario normal, y están enriquecidos por el subtipo luminal B (HER2 +, ER / PR +), además que consisten sólo de los tumores de grado 1 y 2. Los tumores BRCAX-B representan un grupo homogéneo, caracterizado por la pérdida de expresión de "supresor tumoral" miRNA Cluster 2. Los tumores BRCAX-C mostraron un perfil de expresión del los miRNAs similar al de los tumores BRCA1 / 2 y se agrupaban en la misma rama. Estos tumores, se caracterizan por la pérdida del "supresor tumoral" miRNA Cluster 2 y la pérdida de miRNA Cluster 3 relacionado con las funciones migratorias, y la sobreexpresión del miRNA Cluster 3 asociado con el metabolismo de las macromoléculas. Los tumores BRCAX-D carecían de una firma de expresión de los miRNAs específica, y fueron dispersados por todo el clúster. Su perfil de expresión de miRNA se asemeja al de los tumores de mama esporádicos.

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APPENDIX

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Histopathological data of fresh frozen hereditary breast tumor series.

Number	ID	Germline mutation	Lymph node	Grade	ER	PR	HER2	Molecular Subtype
1	01T163	BRCA1	Positive	3	-	-	-	Triple Negative
2	01T167	BRCA1	Negative	3	-	-	-	Triple Negative
3	01T185	BRCA1	Positive	3	-	-	-	Triple Negative
4	02T124	BRCA1	Negative	3	-	-	-	Triple Negative
5	02T144	BRCA1	NA	NA	NA	NA	NA	NA
6	05T126	BRCA1	NA	3	-	-	-	Triple Negative
7	01T265	BRCA2	NA	NA	NA	NA	NA	NA
8	05T312	BRCA2	Positive	2	+	-	-	Luminal A
9	09T155	BRCA2	NA	NA	NA	NA	NA	NA
10	01T248	BRCAX	NA	NA	+	+	+	Luminal B
11	01T306	BRCAX	Negative	1	+	+	-	Luminal A
12	02T328	BRCAX	Negative	3	-	-	+	HER2+
13	04T59	BRCAX	NA	NA	NA	NA	NA	NA
14	05T129	BRCAX	Negative	2	+	-	+	Luminal B
15	05T132	BRCAX	NA	NA	NA	NA	NA	NA
16	05T134	BRCAX	NA	3	+	-	-	Luminal A
17	05T136	BRCAX	Negative	1	+	+	-	Luminal A
18	09T154	BRCAX	NA	NA	NA	NA	NA	NA
19	09T79	BRCAX	Negative	2	-	-	+	HER2+

NA: Not available data; (+) – Positive; (-) - Negative

Supplementary table 2. Clinico-pathological information for FFPE hereditary breast tumor series included in the array analysis

#	Tumor	Mutated Gene	Age at diagnosis	Histological type	Lymph Node	Grade	ER	PR	HER2/ ErbB2	EGFR	CK5 (10%)	p53 (25%)	MIB1/ Ki-67	IHC-based molecular subtype
1	12908	BRCA1	NA	NA	NA	NA	0	0	0	0	1	1	2	Triple Negative
2	01T154	BRCA1	51	DIC	1	3	0	0	0	1	1	0	3	Triple Negative
3	01T163	BRCA1	35	DIC	1	3	0	0	0	NA	1	1	2	Triple Negative
4	01T167	BRCA1	32	DIC	0	3	0	0	0	1	1	1	1	Triple Negative
5	01T185	BRCA1	38	DIC	1	3	0	0	0	1	1	1	2	Triple Negative
6	02T124	BRCA1	55	DIC	0	3	0	0	0	0	1	0	2	Triple Negative
7	02T19	BRCA1	47	DIC	1	2	1	1	0	0	0	0	1	Luminal A
8	03T158	BRCA1	30	DIC	0	3	0	0	0	0	1	1	3	Triple Negative
9	03T326	BRCA1	47	DIC	0	3	1	1	0	0	0	0	1	Luminal A
10	04T270	BRCA1	NA	NA	NA	3	0	0	0	1	0	0	2	Triple Negative
11	04T327	BRCA1	NA	DIC	0	3	0	0	0	1	1	NA	0	Triple Negative
12	04T374	BRCA1	NA	NA	NA	3	0	0	0	0	0	1	3	Triple Negative
13	04T39	BRCA1	28	DIC	1	3	0	0	0	1	1	1	3	Triple Negative
14	02T59	BRCA2	35	DIC	1	3	1	1	0	0	1	1	3	Luminal A
15	03T162	BRCA2	45	LIC	NA	2	0	0	0	0	0	1	2	Triple Negative
16	03T168	BRCA2	46	DIC	1	1	0	1	0	0	0	0	1	Luminal A
17	03T238	BRCA2	37	DIC	1	1	1	0	0	0	NA	0	2	Luminal A
18	03T239	BRCA2	56	DIC	1	3	0	0	0	0	0	0	1	Triple Negative
19	04T309	BRCA2	36	NA	0	3	1	1	0	0	0	NA	3	Luminal A
20	04T328	BRCA2	NA	DIC	0	3	1	1	0	0	0	NA	NA	Luminal A
21	04T329	BRCA2	NA	DIC	0	2	1	1	0	0	0	0	1	Luminal A
22	10T126	BRCA2	NA	NA	NA	2	1	1	1	NA	NA	NA	NA	Luminal B
23	10T171	BRCA2	NA	NA	NA	3	1	1	1	NA	NA	NA	NA	Luminal B
24	00T43	BRCAX	58	DIC	1	3	1	0	NA	NA	0	0	1	NA
25	00T54	BRCAX	49	DIC	1	2	0	0	0	0	0	0	1	Triple Negative
26	01T243	BRCAX	48	DIC	NA	2	0	1	0	NA	NA	NA	NA	Luminal A
27	02T31	BRCAX	68	NA	1	2	0	0	0	0	0	0	1	Triple Negative
28	02T343	BRCAX	NA	DIC	NA	2	1	1	NA	NA	NA	0	1	NA

29	02T37	BRCAX	58	DIC	1	3	0	0	0	1	1	0	2	Triple Negative
30	02T39	BRCAX	47	DIC	0	1	1	1	0	0	0	0	1	Luminal A
31	02T41	BRCAX	34	DIC	1	3	1	0	0	0	0	1	1	Luminal A
32	04T35	BRCAX	50	DIC	NA	1	0	0	0	0	0	0	1	Triple Negative
33	04T355	BRCAX	49	DIC	NA	3	1	1	0	0	0	0	1	Luminal A
34	04T400	BRCAX	NA	NA	NA	2	0	0	0	0	0	0	3	Triple Negative
35	04T55	BRCAX	45	DIC	NA	1	0	0	1	0	0	1	2	HER2
36	06T224	BRCAX	51	NA	0	2	1	0	0	0	NA	NA	NA	Luminal A
37	06T225	BRCAX	35	DIC	1	3	0	0	0	0	NA	NA	NA	Triple Negative
38	06T226	BRCAX	38	DIC	1	3	1	1	0	0	0	0	1	Luminal B
39	06T230	BRCAX	95	DIC	0	2	1	1	0	0	0	0	1	Luminal A
40	06T232	BRCAX	73	DIC	1	3	1	1	0	0	0	1	2	Luminal A
41	06T238	BRCAX	39	DIC	0	3	1	0	1	0	0	1	3	Luminal B
42	06T241	BRCAX	47	DIC	1	3	1	1	1	0	0	1	2	Luminal B
43	06T243	BRCAX	64	NA	1	2	1	0	0	0	0	0	1	Luminal A
44	06T246	BRCAX	55	DIC	1	3	0	0	0	0	0	0	1	Triple Negative
45	06T250	BRCAX	52	DIC	0	2	1	1	0	0	0	0	1	Luminal A
46	06T251	BRCAX	58	DIC	0	3	0	0	0	0	0	1	2	Triple Negative
47	06T253	BRCAX	37	DIC	0	NA	0	0	0	0	0	0	1	Triple Negative
48	06T255	BRCAX	59	DIC	1	2	0	0	1	0	0	0	2	HER2
49	06T256	BRCAX	33	DIC	1	2	1	1	0	0	0	0	1	Luminal A
50	06T258	BRCAX	40	NA	1	3	0	0	0	0	1	1	2	Triple Negative
51	06T260	BRCAX	26	Intraductal	0	3	NA	NA	0	0	0	NA	NA	NA
52	06T264	BRCAX	37	DIC	1	3	0	1	0	0	0	0	2	Luminal A
53	10T123	BRCAX	25	DIC	1	2	0	0	0	NA	NA	NA	NA	Triple Negative
54	10T124	BRCAX	31	Intraductal	0	2	NA	NA	NA	NA	NA	NA	NA	NA
55	10T125	BRCAX	37	Intraductal	0	2	NA	NA	1	NA	NA	NA	NA	NA
56	10T127	BRCAX	55	DIC	0	2	0	0	1	0	NA	1	3	HER2
57	10T136	BRCAX	44	DIC	0	2	1	1	0	0	NA	0	2	Luminal A
58	10T137-2	BRCAX	49	DIC	0	3	0	0	0	0	1	1	3	Triple Negative
59	10T139	BRCAX	48	DIC	0	2	1	1	1	NA	NA	NA	NA	Luminal B

60	10T172	BRCAX	39	Intraductal	NA	1	NA	NA	0	NA	NA	NA	NA	NA
61	11T3	BRCAX	40	DIC	1	3	NA	1	0	NA	NA	NA	NA	Luminal A
62	11T4	BRCAX	54	LIC	1	2	0	0	NA	NA	NA	NA	NA	NA
63	11T71-1	BRCAX	52	DIC	0	2	1	0	1	NA	NA	0	2	Luminal B
64	11T71-2	BRCAX	52	DIC	0	2	1	0	1	NA	NA	0	2	Luminal B
65	11T72	BRCAX	NA	NA		NA	NA	NA	NA	NA	NA	NA	NA	NA
66	11T73	BRCAX	39	DIC	0	3	0	0	0	NA	1	1	3	Triple Negative

ER- estrogen receptor, **PR** - progesterone receptor, **HER2/ErbB2**- human epidermal growth factor; **DIC** - ductal infiltrating carcinoma; **LIC** - lobular infiltrating carcinoma; **NA** - not available; (**0)** - negative; (**1)** - positive;

*Due to the size of the "**Supplementary figure 3**. Full list of differentially expressed genes is shown in HCC1937 vs. HCC1937/BRCA1^{wt} ", it is available only in the electronic form in the attached CD.

Supplementary Table 4. List of pathways significantly associated (FDR<0.05) to DEmiR-DEG pairs

Ingenuity Canonical Pathways	-log(p-value)	Ratio	Molecules
p38 MAPK Signaling	2.79E00	5.21E-02	FADD, TRAF2, IL1F9, IRAK3, IRAK1
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.7E00	2.64E-02	FZD8, TRAF2, IL1F9, CAMK2D, WNT3, C1S, LTB, IRAK3, IRAK1
Role of PKR in Interferon Induction and Antiviral Response	2.3E00	6.67E-02	FADD, TRAF2, RNASEL
NF-κB Signaling	2.04E00	3.29E-02	TRAF2, IL1F9, RELB, IRAK3, IRAK1
Lymphotoxin β Receptor Signaling	1.91E00	4.92E-02	TRAF2, RELB, LTB
Acute Phase Response Signaling	1.77E00	2.81E-02	TRAF2, IL1F9, SOD2, C1S, IRAK1
Basal Cell Carcinoma Signaling	1.71E00	4.41E-02	FZD8, WNT3, BMP7
Human Embryonic Stem Cell Pluripotency	1.65E00	2.7E-02	FZD8, NTF4, WNT3, BMP7
Hepatic Cholestasis	1.52E00	2.4E-02	TRAF2, IL1F9, IRAK3, IRAK1
Factors Promoting Cardiogenesis in Vertebrates	1.44E00	3.37E-02	FZD8, WNT3, BMP7
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	1.44E00	3.45E-02	IL1F9, RELB, LTB
Retinoic acid Mediated Apoptosis Signaling	1.43E00	4.65E-02	FADD, DAP3
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	1.43E00	2.19E-02	FZD8, TRAF2, IL1F9, WNT3, BMP7
PPAR Signaling	1.37E00	3.06E-02	TRAF2, IL1F9, SCAND1
SAPK/JNK Signaling	1.33E00	3.06E-02	FADD, TRAF2, DUSP8

Supplementary Table 5. Cluster 1 - differentially expressed miRNAs (FDR<0.05) in each BRCAX-subgroup in comparison to normal breast tissue

miRNA	Genomic location	BRCAX-A		BRCAX-B		BRCAX-C		BRCAX-D	
		FDR	FC	FDR2	FC2	FDR3	FC3	FDR4	FC4
hsa-let-7a*	09q22.2/11q24.1/22q13.3	NS	0.81	0.0042	1.04	1E-06	0.61	4E-05	0.67
hsa-miR-609	10q25.1	NS	0.64	NS	0.83	2E-05	0.47	3E-05	0.50
hsa-miR-483-3p	11p15.5	0.04401	0.56	NS	2.50	0.0402	0.60	0.0044	0.59
hsa-miR-1237	11q13.1	NS	0.86	NS	1.48	NS	0.77	NS	0.86
hsa-miR-1279	12q15	NS	1.07	NS	1.39	NS	0.90	NS	1.06
hsa-miR-618	12q21.31	NS	0.64	NS	0.87	2E-05	0.45	2E-05	0.49
hsa-miR-625*	**14q23.3**	NS	0.96	NS	0.70	0.0139	1.50	NS	1.18
hsa-miR-493*	**14q32.2**	NS	0.66	NS	0.93	0.0003	0.53	0.0001	0.55
hsa-miR-453	**14q32.31**	NS	0.66	NS	0.87	7E-05	0.51	3E-05	0.51
hsa-miR-1282	15q15.3	NS	1.01	NS	1.92	NS	0.81	NS	0.89
hsa-miR-190	15q22.2/1q21.3/11q12.2	NS	0.71	NS	1.04	0.0001	0.51	0.0001	0.57
hsa-miR-422a	15q22.31	NS	0.70	NS	1.06	0.0017	0.64	0.0002	0.64
hsa-miR-940	16p13.3	NS	0.86	NS	1.94	NS	1.06	NS	0.93
hsa-miR-140-5p	**16q22.1**	NS	0.74	NS	1.14	NS	0.50	NS	0.58
hsa-miR-328	**16q22.1**	NS	0.93	NS	1.57	NS	0.88	NS	0.93
hsa-miR-132*	17p13.3	NS	0.69	NS	1.49	0.0019	0.54	0.0002	0.55
hsa-miR-122	18q21.31	NS	0.90	NS	2.09	NS	0.66	NS	0.73
hsa-miR-1238	**19p13.2**	NS	0.87	NS	1.40	NS	0.81	NS	0.83
hsa-miR-1227	**19p13.3**	NS	0.90	NS	1.61	NS	0.86	NS	0.88
hsa-miR-643	**19q13.41**	NS	0.74	NS	1.25	0.001	0.54	0.0008	0.60
hsa-miR-99b*	**19q13.41**	NS	0.71	NS	1.15	NS	0.77	0.0003	0.64
hsa-miR-373	**19q13.42**	0.0485	0.61	NS	0.75	2E-05	0.45	2E-05	0.50
hsa-miR-517c	**19q13.42**	NS	0.69	NS	0.78	0.0003	0.54	0.0003	0.58
hsa-miR-519c-3p	**19q13.42**	NS	0.95	NS	1.60	NS	0.79	NS	0.85

hsa-miR-519d	**19q13.42**	NS	0.73	NS	1.78	NS	0.66	0.0003	0.55
hsa-miR-520d-3p	**19q13.42**	NS	0.73	NS	1.20	0.0008	0.56	0.0001	0.58
hsa-miR-520h	**19q13.42**	NS	0.87	NS	1.37	0.0228	0.73	NS	0.78
hsa-miR-522	**19q13.42**	NS	0.74	NS	1.30	0.0034	0.64	0.0004	0.66
hsa-miR-526b	**19q13.42**	NS	0.91	NS	1.43	0.0341	0.77	NS	0.82
hsa-miR-942	1p13.1	NS	0.84	NS	1.67	0.0123	0.66	NS	0.72
hsa-miR-190b	1q21.3	NS	1.10	NS	1.29	0.0036	0.73	NS	0.82
hsa-miR-9	1q22/5q14.3/**15q26.1**	NS	0.66	NS	1.10	7E-05	0.44	8E-05	0.50
hsa-miR-135b	1q32.1	NS	0.76	NS	1.25	0.0023	0.58	0.0021	0.62
hsa-miR-181b	1q32.1/9q33.3	NS	0.86	NS	1.19	0.0017	0.66	0.0032	0.70
hsa-miR-664	1q41	NS	0.82	NS	1.31	NS	0.81	0.0002	0.71
hsa-miR-1281	**22q13.2**	0.04028	0.58	NS	2.15	NS	0.67	0.0044	0.64
hsa-let-7b*	**22q13.3**	NS	0.96	0.0044	1.71	7E-06	0.89	3E-05	0.91
hsa-miR-1249	**22q13.31**	NS	0.68	NS	1.92	0.0168	0.56	0.001	0.54
hsa-miR-885-5p	3p25.3	NS	0.98	NS	2.25	NS	1.02	NS	1.02
hsa-miR-302a	4q25	NS	0.71	NS	0.78	3E-05	0.55	2E-05	0.58
hsa-miR-578	4q32.3	NS	0.64	NS	1.29	0.0001	0.41	6E-05	0.47
hsa-miR-581	5q11.2	NS	0.64	NS	1.24	0.0002	0.39	0.0001	0.45
hsa-miR-1294	5q33.2	NS	0.91	NS	1.42	NS	0.80	NS	0.85
hsa-miR-877*	6p21.33	NS	0.65	NS	1.62	0.0111	0.58	0.0005	0.57
hsa-miR-196b	7p15.2	NS	0.76	NS	0.90	0.0001	0.53	0.0001	0.59
hsa-miR-592	7q31.33	NS	0.65	NS	1.48	0.0013	0.43	0.0001	0.49
hsa-miR-593	7q32.1	NS	0.76	NS	1.13	0.0023	0.60	0.0015	0.63
hsa-miR-29a*	7q32.3	NS	1.05	0.0044	1.52	< 1E-08	0.89	1E-04	0.94
hsa-miR-29b-1*	7q32.3	NS	0.79	NS	1.24	0.0008	0.60	0.0003	0.68
hsa-miR-1207-3p	8q24.21	NS	1.14	NS	2.08	NS	1.00	NS	1.07
hsa-miR-30b*	8q24.22	NS	0.88	0.0118	1.53	5E-06	0.77	NS	0.73
hsa-miR-935	9q13.42	NS	0.76	NS	1.98	NS	0.85	NS	0.77

hsa-miR-7	9q21.32/**15q26.1**/9p13.3	NS	1.30	0.0328	1.98	0.0035	1.36	0.0035	1.36
hsa-miR-601	9q33.3	NS	0.70	NS	1.13	0.0021	0.61	0.0005	0.62
hsa-miR-188-5p	Xp11.23	NS	0.72	NS	0.82	0.0002	0.56	0.0001	0.60
hsa-miR-221	Xp11.3	NS	0.77	NS	0.93	0.0054	0.61	0.0019	0.66
hsa-miR-651	Xp22.31	NS	0.78	NS	1.27	0.0023	0.68	0.0001	0.70
hsa-miR-548m	Xq21.33	NS	0.90	NS	1.25	0.0157	0.74	NS	0.80
hsa-miR-450a	Xq26.3/	NS	0.71	NS	1.08	0.0004	0.56	0.0001	0.59
hsa-miR-508-3p	**Xq27.3**	NS	0.80	NS	1.04	0.0002	0.62	6E-05	0.65
hsa-miR-892b	**Xq27.3**	NS	0.71	NS	0.93	0.0002	0.52	0.0001	0.55
hsa_SNORD12				NS	1.39	0.0484	0.73	NS	0.71

Supplementary Table 6. Cluster 2 differentially expressed miRNAs (FDR<0.05) in each BRCAX-subgroup in comparison to normal breast tissue

miRNA	Genomic location	BRCAX-A		BRCAX-B		BRCAX-C		BRCAX-D	
		FDR	FC	FDR2	FC2	FDR3	FC3	FDR4	FC4
hsa-let-7i	12q14.1	NS	0.80	0.00448	0.51	< 1.0E-08	0.31	0.0000918	0.48
hsa-let-7c	21q21.1	NS	0.89	0.02861	0.33	0.000857	0.46	0.0023494	0.57
hsa-let-7b	22q13.31	NS	0.92	0.00437	0.29	0.000007	0.30	0.0000258	0.44
hsa-let-7g	3p21.1	NS	0.98	0.00264	0.39	< 1.0E-08	0.28	0.0001043	0.53
hsa-let-7a	**9q22.2**/11q24.1/22q13.3	NS	0.98	0.00424	0.29	1.2E-06	0.22	0.0000401	0.46
hsa-let-7f	**9q22.2**/Xp11.22	NS	1.40	0.01205	0.45	2.42E-05	0.25	0.0044077	0.56
hsa-let-7d	**9q22.32**	NS	1.05	0.0054	0.38	4.83E-05	0.34	0.0039411	0.58
hsa-miR-101	1p31.3/9p21.1	NS	0.88	NS	0.33	NS	0.37	NS	0.53
hsa-miR-125b	11q24.1/21q21.1	0.00115	0.34	0.00066	0.12	< 1.0E-08	0.08	0.0000041	0.16
hsa-miR-126	9q34.3	NS	0.54	NS	0.23	< 1.0E-08	0.19	NS	0.31
hsa-miR-130a	11q12.1	NS	0.59	0.00019	0.23	< 1.0E-08	0.25	0.0002739	0.37
hsa-miR-143	5q32	0.04401	0.58	0.0035	0.21	< 1.0E-08	0.13	0.0000041	0.24
hsa-miR-15a	13q14.2	NS	1.33	NS	1.06	0.000194	0.56	NS	0.87
hsa-miR-195	17p13.1	NS	0.62	0.00474	0.22	1.2E-06	0.23	0.0001118	0.31
hsa-miR-15b	3q25.33	NS	1.09	NS	0.75	0.003658	0.71	NS	0.92
hsa-miR-16	17p13.1/3q25.33	NS	1.48	0.03115	0.36	0.000739	0.38	NS	0.81
hsa-miR-17	13q31.3	NS	0.99	NS	0.76	0.000568	0.65	NS	0.97
hsa-miR-106b	**7q22.1**	NS	1.55	0.01511	0.55	0.00235	0.65	NS	0.93
hsa-miR-106a	Xq26.2	NS	0.98	0.00772	0.65	0.00029	0.65	NS	0.95
hsa-miR-191	3p21.31	0.00115	3.28	0.04451	0.64	0.038274	0.72	NS	1.07
hsa-miR-193a-3p	17q11.2	NS	1.76	NS	0.73	NS	0.69	NS	1.01
hsa-miR-199a-3p	**19p13.2**	NS	0.97	0.00448	0.45	0.000068	0.48	NS	0.70
hsa-miR-199a-5p	**19p13.2**	NS	0.82	0.00038	0.39	< 1.0E-08	0.31	0.0010598	0.48
hsa-miR-22	17p13.3	NS	1.54	0.02104	0.44	0.001856	0.51	NS	0.98

hsa-miR-222	Xp11.3	NS	0.75	0.00683	0.51	1.95E-05	0.49	NS	0.70
hsa-miR-23a	**19p13.13**	NS	1.09	0.00836	0.37	0.000005	0.32	NS	0.70
hsa-miR-23b	**9q22.32**	NS	1.03	0.00264	0.42	1.4E-06	0.37	NS	0.64
hsa-miR-24	**9q22.32/19p13.13**	NS	1.06	0.02104	0.54	1E-07	0.29	NS	0.68
hsa-miR-26b	2q35	NS	0.95	0.02697	0.28	9E-07	0.21	0.0001849	0.42
hsa-miR-26a	3p22.2/12q14.1	NS	0.87	0.0054	0.19	1E-07	0.15	0.0000401	0.30
hsa-miR-29c	1q32.2	NS	1.61	NS	0.53	0.016764	0.58	NS	0.93
hsa-miR-29a	**7q32.3**	NS	0.80	0.00436	0.22	< 1.0E-08	0.16	0.0000972	0.41
hsa-miR-29b	**7q32.3**/1q32.2	NS	1.71	NS	0.77	NS	0.75	NS	1.17
hsa-miR-30e	1p34.2	NS	1.50	NS	0.85	0.011948	0.70	NS	0.96
hsa-miR-30a	**6q13**	0.00903	2.05	0.00297	0.26	4.5E-06	0.26	NS	0.61
hsa-miR-30c	**6q13**/1p34.2	NS	1.04	0.03909	0.59	< 1.0E-08	0.30	0.0000401	0.47
hsa-miR-30b	**8q24.22**	NS	1.64	0.01184	0.27	4.5E-06	0.26	NS	0.52
hsa-miR-30d	**8q24.22**	NS	1.21	NS	0.82	6.9E-06	0.56	0.0005759	0.67
hsa-miR-34a	1p36.22	NS	1.07	0.00772	0.56	0.00068	0.62	NS	0.81
hsa-miR-374a	Xq13.2	NS	1.42	NS	0.68	0.004224	0.68	NS	0.89
hsa-miR-768-3p	snoRNA, HBII-239	NS	1.20	0.03277	0.48	0.000787	0.49	NS	0.85
hsa-miR-141	12p13.31	0.00122	2.71	NS	0.72	0.014291	0.56	NS	1.07
hsa-miR-200c	12p13.31	0.00442	2.22	0.00683	0.49	0.020487	0.63	NS	0.96
hsa-miR-200a	1p36.33	0.00115	2.19	NS	0.82	NS	0.79	NS	1.10
hsa-miR-200b	1p36.33	0.00115	2.21	NS	0.71	0.019219	0.67	NS	1.08
hsa-miR-1264	Xq23	NS	1.72	NS	0.68	NS	1.00	NS	1.28
hsa-miR-93	7q22.1	NS	1.39	NS	0.92	0.015206	0.78	NS	1.03
hsa_SNORD10		NS	1.23	NS	0.66	0.00128	0.59	NS	0.99

Supplementary Table . Cluster 3 differentially expressed miRNAs (FDR<0.05) in each BRCAX-subgroup in comparison to normal breast tissue

miRNA	Genomic location	BRCAX-A		BRCAX-B		BRCAX-C		BRCAX-D	
		FDR	FC	FDR2	FC2	FDR3	FC3	FDR4	FC4
hsa-miR-938	10p11.23	NS	1.34	NS	0.93	2.03E-05	2.68	NS	1.67
hsa-miR-146b-3p	10q24.32	NS	1.25	NS	0.93	0.000442	1.69	3.3E-05	1.53
hsa-miR-202	10q26.3	NS	1.03	NS	1.08	0.004028	1.95	NS	1.17
hsa-miR-483-5p	**11p15.5**	NS	0.98	NS	0.81	0.000777	2.01	NS	1.48
hsa-miR-485-3p	**11p15.5**	NS	1.21	NS	0.89	0.003089	1.62	0.00013	1.66
hsa-miR-675	**11p15.5**	NS	0.66	NS	0.50	0.019869	1.95	NS	0.82
hsa-miR-1261	11q14.3	NS	1.21	NS	0.91	0.000487	1.85	0.00027	1.64
hsa-miR-920	12p12.1	NS	1.05	NS	0.89	0.012382	1.35	NS	1.25
hsa-miR-492	12q22	NS	0.92	NS	0.87	0.005645	1.70	NS	1.08
hsa-miR-617	12q32.31	NS	1.01	NS	0.90	0.000137	2.30	NS	1.39
hsa-miR-623	13q32.3	NS	0.81	NS	0.83	0.049947	1.45	NS	0.81
hsa-miR-208a	14q11.2	NS	1.16	NS	0.93	0.000777	2.15	0.00381	1.46
hsa-miR-381	**14q32.31**	NS	1.10	NS	0.86	0.00211	2.02	NS	1.31
hsa-miR-299-3p	**14q32.31**	NS	0.96	NS	0.86	0.025988	1.40	NS	1.15
hsa-miR-323-3p	**14q32.31/14q32.31**	NS	0.95	NS	0.90	0.048284	1.31	NS	1.06
hsa-miR-494	**14q32.31/14q32.31**	NS	1.98	NS	1.00	0.000166	4.79	NS	2.58
hsa-miR-628-3p	15q21.3	NS	1.63	NS	1.03	3.21E-05	3.38	0.00042	2.27
hsa-miR-184	15q25.1	NS	1.03	NS	1.14	6.7E-06	2.09	0.00338	1.33
hsa-miR-549	15q25.1	0.04401	1.74	NS	1.11	3.5E-06	3.55	6.2E-06	2.58
hsa-miR-423-3p	17q11.2	NS	1.02	NS	0.72	NS	1.23	NS	1.08
hsa-miR-423-5p	17q11.2	NS	0.86	NS	0.75	0.000347	1.95	NS	1.04
hsa-miR-638	19p13.2	NS	0.68	NS	0.47	NS	1.15	NS	0.58
hsa-miR-498	**19q13.42**	NS	0.88	NS	0.65	0.005846	1.63	NS	0.94
hsa-miR-516a-5p	**19q13.42**	NS	1.58	NS	0.78	1.44E-05	4.65	NS	2.12

hsa-miR-516b	**19q13.42**	NS	1.11	NS	0.99	8.63E-05	2.34	NS	1.57
hsa-miR-525-5p	**19q13.42**	NS	0.84	0.03404	0.60	0.003556	1.59	NS	0.94
hsa-miR-518a-5p	**19q13.42/19q13.42**	NS	1.04	NS	0.64	0.002269	2.06	NS	1.28
hsa-miR-518e*/hsa-miR-519a*/hsa-miR-519b-5p/hsa-miR-519c-5p/hsa-miR-522*/hsa-miR-523*	**19q13.42**	NS	0.94	NS	0.73	NS	1.85	NS	1.21
hsa-miR-519e*	**19q13.42**	NS	0.99	NS	0.67	0.000913	2.21	NS	1.17
hsa-miR-552	1p34.3	NS	1.12	NS	0.92	0.000083	2.15	NS	1.51
hsa-miR-551a	1p36.32	NS	0.82	NS	0.97	0.012946	1.33	NS	0.91
hsa-miR-765	1q23.1	NS	0.98	NS	1.16	0.000388	1.87	NS	1.44
hsa-miR-921	1q24.1	NS	1.23	NS	1.03	7.76E-05	2.50	0.00047	1.86
hsa-miR-557	1q24.2	NS	0.71	0.03277	0.55	0.020576	2.07	NS	0.94
hsa-miR-215	1q41	NS	1.02	NS	0.91	0.004186	1.54	NS	1.12
hsa-miR-659	22q13.1	NS	0.74	NS	0.67	NS	1.23	NS	0.80
hsa-miR-933	2q31.1	NS	1.36	NS	0.67	0.00021	2.86	NS	1.53
hsa-miR-1284	3p13	NS	1.04	NS	0.83	0.006484	1.55	NS	1.23
hsa-miR-198	3q13.33	NS	0.78	NS	0.72	0.015405	1.47	NS	0.94
hsa-miR-574-3p	4p14	NS	1.17	NS	0.88	0.049584	1.39	NS	1.51
hsa-miR-874	5p31.2	NS	0.82	NS	0.72	0.010297	1.87	NS	0.96
hsa-miR-583	5q15	NS	1.04	NS	0.88	0.001657	2.00	NS	1.53
hsa-miR-340	5q35.3	NS	1.29	NS	0.94	0.001647	1.65	0.00038	1.46
hsa-miR-1275	6p21.31	NS	0.60	NS	0.62	0.000332	3.04	NS	1.22
hsa-miR-877	6p21.33	NS	1.10	NS	0.98	0.000325	1.72	NS	1.27
hsa-miR-1285	7q21.2/2p13.3	NS	1.70	NS	1.33	0.000011	3.86	0.00019	2.35
hsa-miR-489	7q21.3	NS	0.98	NS	0.84	0.00013	2.12	NS	1.33
hsa-miR-490-3p	7q33	NS	1.09	NS	0.99	0.001613	1.53	0.00452	1.34
hsa-miR-671-5p	7q36.1	NS	0.82	NS	0.71	0.01849	1.71	NS	0.98

hsa-miR-1273	8q22.2/6q25.2/1p36.22/1p32.3/1p32.3	NS	1.18	NS	1.03	0.000249	1.96	0.00338	1.63
hsa-miR-602	9q34.3	NS	0.93	NS	0.53	0.000204	2.32	NS	1.09
hsa-miR-663	centromere/2q21.2	NS	0.92	NS	0.84	0.005726	1.64	NS	1.00
hsa-miR-542-5p	Xq26.3	NS	0.90	NS	0.86	0.003777	1.72	NS	1.08
hsa-miR-934	Xq26.3	NS	1.09	NS	0.93	0.005483	1.45	0.00027	1.39
hsa-miR-508-5p	**Xq27.3**	NS	1.03	NS	0.85	0.000899	2.17	NS	1.34
hsa-miR-509-3-5p	**Xq27.3**	NS	1.01	NS	0.75	0.002727	1.63	NS	1.18
hsa-miR-510	**Xq27.3**	NS	0.86	NS	0.84	0.020576	1.35	NS	0.98
hsa-miR-513b	**Xq27.3**	NS	1.33	NS	1.18	1.44E-05	2.37	2.9E-05	1.61
hsa-miR-509-5p	**Xq27.3/Xq27.3**	NS	1.17	NS	0.75	0.001856	1.85	NS	1.41
hsa-miR-513a-5p	**Xq27.3/Xq27.3**	NS	1.45	NS	1.32	2.6E-06	3.17	0.00027	2.15
hsa-miR-149*	2q37.3	0.00903	0.64	NS	0.56	NS	1.21	NS	0.68
hsa-miR-183*	7q32.2	NS	1.12	NS	0.81	2.95E-05	2.93	NS	1.57
hsa-miR-187*	18q12.22	NS	1.12	NS	0.80	0.000164	2.52	NS	1.46
hsa-miR-193b*	16p13.12	NS	0.79	0.0211	0.57	NS	1.30	NS	0.90
hsa-miR-196a*	12q13.13	NS	1.19	NS	0.98	0.002269	1.51	NS	1.25
hsa-miR-200b*	1p36.33	NS	2.21	NS	0.71	0.019219	0.67	NS	1.08
hsa-miR-25*	7q22.1	NS	1.10	NS	0.93	1E-07	3.58	0.0003	1.72
hsa-miR-302c*	4q25	NS	0.78	0.01738	0.68	NS	1.17	0.00027	0.73
hsa-miR-30c-1*	**6q13**/1p34.2	NS	0.79	NS	0.80	NS	1.25	NS	0.90
hsa-miR-550*	7p14.3/7p14.3/7p14.3	NS	1.59	NS	1.01	0.009972	1.48	0.00019	1.78
hsa-miR-551b*	3q26.2	NS	1.01	NS	0.85	0.001466	1.88	NS	1.31
hsa-miR-629*	15q23	NS	0.96	NS	0.93	0.038274	1.36	NS	1.07
hsa-miR-92b*	1q22	NS	0.98	NS	0.88	0.011746	1.46	NS	1.07

PUBLICATIONS

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PRECLINICAL STUDY

Integration of BRCA1-mediated miRNA and mRNA profiles reveals microRNA regulation of TRAF2 and NFκB pathway

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Abstract Microarray-based techniques are being useful to obtain miRNA and gene expression signatures associated with different tumors. BRCA1 deregulation is a frequent event in the pathogenesis of breast as well as other cancers. In addition to DNA repair functions of BRCA1, it is involved in a wide range of cellular processes such as cell cycle, chromatin remodeling or transcription. However, the molecular events underlying BRCA1-associated tumorigenesis are still largely unknown. In order to deepen our understanding of *BRCA1*-associated tumorigenesis, we integrated data from mRNA and miRNA microarray experiments on HCC1937 breast cancer cell line, and the isogenic HCC1937 stably expressing *BRCA1*, to obtain significant miRNA–mRNA relationships associated with the presence of BRCA1 gene. By using bioinformatic integration of gene and miRNA expression data, we found significant miRNA–gene relationships underlying the array signatures. We additionally evaluated the role of these statistically significant pairs at the biological pathways level and identified MAPK and NF-κB pathways associated with these expression changes. Furthermore, we experimentally validated miRNAs induced by *BRCA1* that commonly regulate *TRAF2*, a key regulator of NF-κB and MAPK pathways. We demonstrate that miR-146a, miR-99b and miR-205, induced in HCC1937 BRCA1-expressing cells, bind and regulate *TRAF2* gene. In addition, re-expression of miR-146a, miR-99b or miR-205 in HCC1937 *BRCA1*-null cells was sufficient to modulate NF-κB activity. Our results demonstrate that integration of mRNA and miRNA associated with *BRCA1* expression was useful to discover new miRNA–gene interactions as molecular events underlying *BRCA1*-mediated tumorigenesis.

Keywords BRCA1 · Hereditary breast cancer · microRNA · mRNA expression · Data integration · TRAF2 · NF-KappaB

Introduction

Loss of BRCA1 activity is a well-known factor for susceptibility to breast and ovarian cancers [1–6]. Several BRCA1 functions have been demonstrated so far [7, 8], including DNA damage repair/recombination, cell cycle control [9], chromatin remodeling and ubiquitylation [10], and regulation of both transcriptional activation and repression [11, 12].

MicroRNAs (miRNAs) are a class of short non-coding RNA that act predominantly as negative regulators of gene expression at post-transcriptional level, by binding to the 3′UTR of their target genes. miRNAs play critical roles in control of various biological processes and are extensively implicated in cancer pathogenesis [13–17]. Individual miRNAs can have multiple targets, and each mRNA may be regulated by multiple miRNAs. Still, due to the scarcity

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of experimentally validated miRNA target genes, much remains to be discovered about the cellular circuits controlled by miRNAs and their biological role. The combined effects of a miRNA on multiple targets emphasize the need to examine the net result of the complex modulation of multiple targets belonging to multiple pathways.

Breast cancer is a molecularly heterogeneous disease that microarray expression studies have helped to dissect. Similar to mRNA expression profiling, abnormal miRNA expression has previously been shown associated with the different molecular breast cancer subtypes [18, 19]. Therefore, integrative studies trying to establish correlative relationships between miRNAs and mRNA expression would improve our understanding of the role of miRNAs in control biological networks in cancer. Some studies have tried to jointly analyze miRNA expression data with its matching mRNA data to better understand the role of miRNA in breast cancer pathogenesis and progression [18].In addition, integration of miRNA and mRNA expression data has been useful to establish an important role for miRNAs in regulating particular cellular processes such as proliferation, cell adhesion or immune response in primary breast cancer [20].

Regarding *BRCA1*-induced tumorigenesis, large-scale genomic approaches have identified relevant genes important for tumorigenesis in relation to *BRCA1* expression [21–27]. However, no studies so far have looked into BRCA1-mediated regulation of miRNA expression. In this study, we sought to identify miRNAs and miRNA–mRNA networks dependent on *BRCA1* expression, by performing an integration analysis of genome-wide miRNA and gene expression data, in isogenic breast cancer cell lines differing in *BRCA1* expression status. Functional validation revealed miRNA regulation of new genes and pathways that might be important in *BRCA1*-related breast tumors.

Materials and methods

Breast cancer cell lines and tissue samples

Paired isogenic cell lines deficient and proficient for *BRCA1* expression were used: HCC1937 derived from hereditary *BRCA1*-mutated breast tumor and HCC1937/BRCA1^{wt} stably expressing full-length *BRCA1*, which were kindly provided by Dr. J. Chen (Yale University School of Medicine Department of Therapeutic Radiology, New Haven, USA). Cell lines were cultured in DMEM medium supplemented with 10%FBS.

In addition, 13 formalin-fixed paraffin-embedded (FFPE) tumor tissues from hereditary breast cancer cases harboring *BRCA1* mutations, as well as four normal breast tissues from mammary reduction surgery, were selected to check the expression of interesting miRNAs.

Microarray experiments

miRNA expression profiling

Total RNA was extracted from cell lines using Trizol (Invitrogen). RNA from FFPE tumor samples was extracted by using RNeasy FFPE kit (Qiagen). miRNA expression profiling was performed using miRCURY LNA^{TM} microRNA Array kit (Exiqon). Briefly, 300 ng of total RNA was labeled with Hy3 fluorescent dye and subsequently hybridized over 16 h at 56°C onto miRNA microarray chip (v.11.0—hsa, mmu & rno). Fluorescence intensities on scanned images were quantified using Feature Extraction software (Agilent Technologies) using the modified Exiqon protocol. Background correction was carried out, and raw data were normalized by quantiles. Each sample was hybridized in triplicate. Microarray dataset is publicly available at GEO database http://www.ncbi.nlm.nih.gov/geo/info/linking.html (GEO accession number, GSE30822, SubSeries GSE30763).

mRNA expression profiling

For whole-genome transcriptional profiling, 500 ng of total RNA from samples and Universal Human Reference RNA (Stratagene, La Jolla, CA, USA) were used for amplification and labeling using the Low RNA Input Linear Amplification Kit (Agilent Technologies). For each hybridization, 1 μg of cyanine 3-labeled cRNA (reference) and 1 μg of cyanine 5-labeled cRNA (samples) were mixed, fragmented and hybridized at 65°C for 17 h to an Agilent 4 × 44 K Whole Human genome Oligo Microarray. Each sample was hybridized in triplicate. Data were extracted with Agilent feature extraction software (version 9.5.3). Background subtraction was carried out. Lowess and quantiles methods were performed for intra-array and inter-array normalization, respectively. Microarray dataset is publicly available at GEO database (GEO accession number, GSE30822, SubSeries GSE30821).

Microarray data analysis

To determine whether there were mRNA and miRNA genes differentially expressed between BRCA1-deficient and BRCA1-proficient breast cancer cell lines, differential expression analysis was performed with linear models (limma) implemented in the POMELO II tool, available in Asterias web server (http://asterias.bioinfo.cnio.es). The estimated significance level (P value) was corrected for multiple hypotheses testing using Benjamini and Hochberg false discovery rate (FDR) adjustment. Those miRNAs with FDR < 0.05 were selected as significantly differentially expressed, while for mRNAs, FDR < 0.01 was set as threshold.

Integration of miRNA and mRNA gene expression signatures

For each differentially expressed miRNA, a contingency table relating the miRNA and its predicted gene targets was produced using miRBase Targets Release v. 5.0 (http://www.mirbase.org/; Faculty of Life Sciences, University of Manchester), taking into account whether these targets were included in a consistent gene expression signature (down-regulated targets for up-regulated miRNAs and vice versa). Fisher's exact test was used for significance analysis. Those miRNAs whose Fisher's exact test had $P < 0.05$ were selected for further analysis. To identify statistically significant associations between differentially expressed miRNAs (both t-test and Fisher's exact test $P < 0.05$) and enriched pathways (FDR < 0.05), a ranked list was built. Down-regulated miRNAs were tested for their association with up-regulated genes, whereas up-regulated miRNAs were tested for their association with down-regulated genes. The ranked target list of the differentially expressed genes was subjected to pathway enrichment analyses using Ingenuity Pathway Analysis 7.6 software (Ingenuity Systems, Redwood City, CA). Interaction networks were built and depicted using Cytoscape bioinformatics software (http://www.cytoscape.org).

Real-time PCR analysis

Quantitative RT-PCR analysis of miRNA expression was performed using miRCURY LNA™ microRNA PCR System (Exiqon). Briefly, 10 ng of total RNA was reverse-transcribed with miRNA-specific primers and transcriptor reverse transcriptase, and miRNA-specific LNA™ PCR primer and Universal PCR primer were used for the PCR. Gene expression levels were quantified using the ABI Prism Sequence Detection System 7900HT (Applied Biosystems). Normalization was done with SNORA66 RNA and 5S rRNA.

To detect gene expression levels of *TRAF2*, 1 µg of total RNA was reverse-transcribed using MMLV Reverse Transcriptase (Invitrogen) and random primers. The cDNAs were subjected to quantitative RT-PCR assay with the use of labeled probes (Roche Universal Probe library) and TaqMan Universal PCR Mix in an ABI Prism Sequence Detection System 7900HT (Applied Biosystems) using the following oligonucleotides (F:5′GCATACCGC CATCTTCTC3′ and R:5′CGTTCAGGTAGATACGCAG ACA3′). ß-Actin was used as internal control and allowed for normalization of the samples. All experiments were analyzed in triplicate. Relative expression was calculated using the comparative Ct method.

Cell-based multi-pathway activity assays

Using Cignal Multi-Pathway Reporter Assay (SABiosciences, QIAGEN), we evaluated activation of ten different cancer-related pathways. Briefly, the HCC1937 and HCC1937/BRCA1 were transfected with the transcription factor-responsive reporter, negative control and positive control constructs using SureFECT™ (SABiosciences). The change in the activity of each signaling pathway is determined by comparing the normalized luciferase activities of the reporter in *BRCA1*-null versus *BRCA1*-wt expressing HCC1937 cells.

For assessment of miRNA effect on pathway activation, 200 pmol of pre-miR-146a, pre-miR-99b, pre-miR-205 or negative control was co-transfected along with pathway reporter in HCC1937 cells. The transfection efficiency was monitored by GFP expression, using fluorescence microscopy. At least three independent transfections were carried out in triplicate for each of the conditions tested with each reporter assay.

Cell transfections and target in vitro assay

Pre-miRNA oligonucleotides (pre-miR-146a, pre-miR-99b, pre-miR-205 and scramble control) were purchased from Ambion (Austin, Texas, USA). For luciferase reporter target in vitro assays, 250 nM OmicsLink miRNA Target Clone (GeneCopoeia, Rockville, MD) containing Renilla luciferase and TRAF2 3′UTR cloned downstream of firefly luciferase gene, together with 50 nM pre-miRNA oligonucleotides, was transfected using Lipofectamine 2000 reagent (Invitrogen, San Diego, CA) into cells in a 24-well plate format. We have co-transfected HCC1937 cells with the *TRAF2* 3′UTR reporter construct along with each individual pre-miRNA (Ambion), or combination of all three miRNAs or scramble control. Cells were grown for 48 h, after which luciferase activity was assayed with Dual-Luciferase Assay System (Promega). Experiments were performed in triplicate, and normalization was performed using Renilla luciferase activity.

For western blot analysis HCC1937 cells were transfected with 50nM pre-miRNA oligonucleotides (miR-146, miR-99 and miR-205). Cells were grown for 48 h and harvested.

Western blotting

Western blot analysis was performed using standard procedure for whole-cell extracts from cell lines. Lysates were prepared using RIPA buffer (Sigma-Aldrich). Equal amounts of protein lysates (50–100 µg) were separated by SDS-PAGE on 4–12% pre-casted gels (Invitrogen),

electrotransferred to nitrocellulose membranes and probed with primary antibody against TRAF2 (C-20, Santa Cruz) and GAPDH (CNIO, Monoclonal antibody Unit).

Statistical analysis

To asses differences between pathway activation levels in the HCC1937 and HCC1937/BRCA1^{wt}, and differences in miRNA-mediated regulation of TRAF2 and MAPK and NF-B pathways by luciferase reporter assay, as well as differential miRNA expression between *BRCA1* tumors and normal breast samples, bilateral t-test was applied using SPSS version 17 (SPSS Inc, Chicago, Illinois). Nominal two-sided *P*-values less than 0.05 were considered statistically significant.

Results

Significantly modulated mRNAs and miRNAs in response to BRCA1 reconstitution

To determine the effect of BRCA1 reconstitution on miRNA and mRNA profiles, we performed whole-genome transcriptional profiling and global miRNA expression profiling in the *BRCA1*-deficient HCC1937 breast cancer cell line and its isogenic counterpart HCC1937/*BRCA1*^{wt}, stably expressing wt *BRCA1*. A total of 8845 genes demonstrated statistically significant (FDR < 0.01) differential expression, representing a 26% shift in global gene expression, with 4710 transcripts down-regulated and 4135 transcripts up-regulated in *BRCA1*-proficient cells relative to *BRCA1*-deficient HCC1937 breast cancer cells (Fig. 1a). The list of these genes is shown in Supplementary Table 1.

On the other hand, miRNA expression profiles exhibited very high similarity in the two isogenic cell lines, with a correlation coefficient of $R^2 = 0.93$ (Fig. 1c). Differential expression analysis showed that only 9 miRNAs (miR-99a, miR-99b, miR-138, miR-146a, miR146b, miR-205, miR-302b*, miR-923 and miR-1290) were significantly differentially expressed between the cell lines with FDR < 0.05, representing 1% change in global miRNA expression (Fig. 1b). Most of these miRNAs were up-regulated in HCC1937/BRCA1^{wt} compared to HCC1937, while only miR-138 was down-regulated in HCC1937/BRCA1^{wt} (Supplementary Table 2). Quantitative RT-PCR analysis performed on selected miRNAs confirmed the pattern of differential expression shown by microarray analysis.

miRNA and mRNA integration analysis reveals significant miRNA–mRNA target pairs and pathways

To identify statistically significant associations between differentially expressed miRNAs (DEmiRs, FDR < 0.05) and differentially expressed genes (DEGs, FDR < 0.01), we tested whether predicted miRNA–mRNA targeting pairs were non-randomly associated [28]. Gene target predictions for human miRNAs were obtained using miRBase Targets Release v5.0 (http://www.miRBase.org, Faculty of Life Sciences, University of Manchester). Results of Fisher's exact test indicated that miR-138, miR-146a, miR-146b and miR-99b were significantly associated with differentially expressed mRNA targets within the gene expression signature. Significant miRNAs up-regulated in HCC1937/BRCA1^{wt} (miR-146a, miR-146b and miR-99b) were collectively targeting 160 down-regulated DEGs. Interestingly, five genes *SURF1, TRAF2, FBXW2, HIST1H4I and MARK1* were identified as common targets for all three significant up-regulated miRNAs. On the other hand, miR-138 was significantly associated with 75 up-regulated DEG.

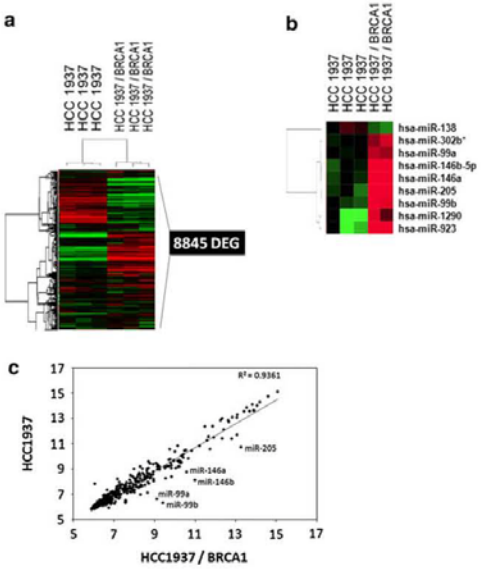

Fig. 1 mRNA and miRNA transcriptional changes upon BRCA1 reconstitution. **a** Differentially expressed genes between HCC1937 and HCC 1937/BRCA1^{wt} cells. mRNA expression heatmap of genes differentially expressed (FDR < 0.01). Up-regulated genes in HCC1937/BRCA1^{wt} are shown in *red* and down-regulated genes in *green*. **b** Differentially expressed miRNAs between HCC1937 and HCC 1937/BRCA1^{wt} cells. miRNA expression heatmap over nine differentially expressed miRNAs (FDR < 0.05). Up-regulated miRNAs in HCC1937/BRCA1^{wt} are shown in *red* and down-regulated in *green*. **c** Correlation between miRNA expression in HCC1937 and HCC 1937/BRCA1^{wt} cells. Linear regression analysis of global miRNA expression in the two isogenic cell lines. Squared correlation coefficient of $R^2 = 0.93$ indicates high similarity of the two datasets

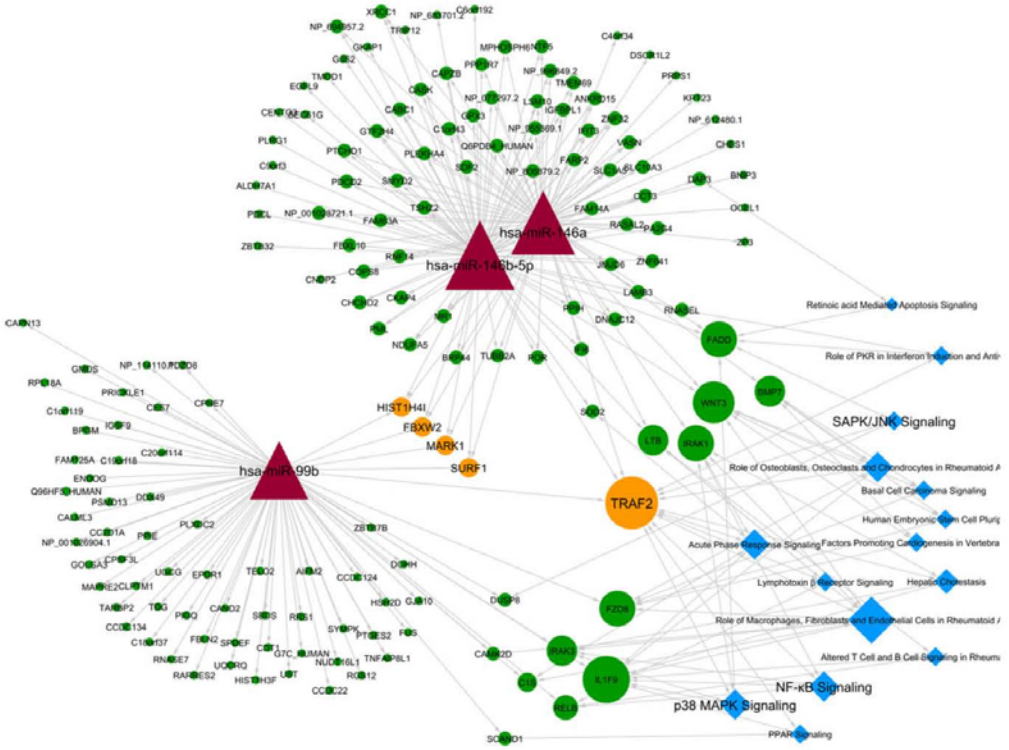

Fig. 2 Up-regulated miRNAs in HCC 1937/BRCA1^{wt} cells with connections to down-regulated genes and pathways. miRNAs are indicated by *red triangles*, miRNA target genes are depicted as *green* (1–2 connections of coding genes to miRNAs) and *yellow circles* (3 connections), whereas pathways are represented by *blue diamonds*. The size is proportional to their degree of connectivity. All the connections represent statistically significant relationships with FDR < 0.05 set as threshold

To explore the biological significance for observed changes, significant miRNA–mRNA target pairs were evaluated for representation of relevant functional biological processes and pathways. The pathway analysis over the 160 significant down-regulated DEG revealed statistically significant enrichment for 15 pathways related to proliferation signaling and inflammation. Among the most interesting were p38/MAPK, SAPK/JNK and NF-κB signaling pathways. Interestingly, *TRAF2* (TNF-receptor-associated factor 2) gene, a well-established mediator of both NF-κB and MAPK pathway activation [29], was predicted to be a target of the three significant miRNAs (miR-146a, miR-146b and miR-99b) along with another two out of the nine differentially expressed miRNAs, miR-99a and miR-205. On the other hand, there were only two pathways (clathrin-mediated endocytosis signaling and IL-10 signaling) that were significantly enriched within 75 significant up-regulated DEG, targets of miR-138. Full list of pathways is represented in Supplementary Table 3.

An interaction network (Fig. 2) was built to represent connections of significant up-regulated DEmiRs, their DE-mRNA targets and the associated pathways. This approach allowed us to infer experimental microarray gene expression data to predict the effects of miRNA expression on the global behavior of biological pathways.

Changes in pathway activation between BRCA1-proficient and BRCA1-deficient HCC1937 cells

We quantitatively assessed signal transduction pathway activation by measuring the activities of downstream transcription factors in vitro in the isogenic cell lines deficient and proficient for BRCA1. Interestingly, the results revealed that MAPK/JNK pathway was significantly down-regulated in HCC1937/BRCA1^{wt} compared to the BRCA1-null HCC1937 cell line (Fig. 3). In addition, NF-κB and MAPK/ERK showed also reduced activity when *BRCA1* was expressed in

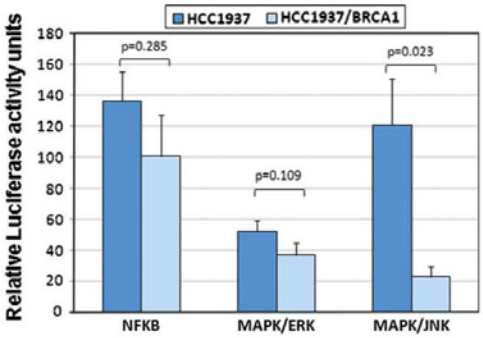

Fig. 3 Luciferase reported assay for activation of signaling pathways. MAPK/ERK, MAPK/JNK and NFκB signaling pathways show decreased activation upon BRCA1 reconstitution. Pathway activation in the BRCA1-deficient and BRCA1-proficient isogenic HCC1937 cell lines was assessed by measuring the activities of downstream transcription factors. Transcription factor (TF)-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal (m) CMV promoter and tandem repeats of the TF transcriptional response element (TRE). Reporter transcription factors used for assessment of NFκB, MAPK/ERK and MAPK/JNK pathway signaling were NFκB, Elk-1/SRF and AP-1, respectively

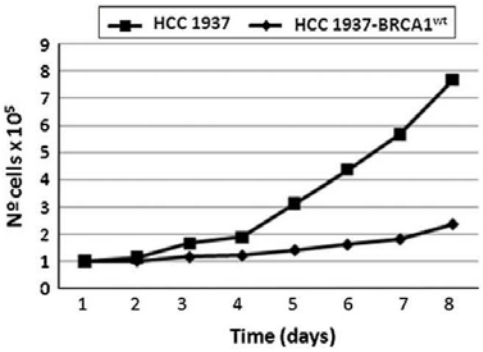

Fig. 4 Growth curves of HCC1937 and HCC1937 BRCA1-expressing cells. BRCA1 reconstitution impacts proliferation of HCC1937 cells. $BRCA1^{wt}$-expressing HCC1937 cells showed a lower increase in the number of growing cells comparing to the HCC1937 BRCA1-null cells

HCC1937 cells, although this was not statistically significant. Reduction in the activity of these pathways at least partially supports the results obtained by Ingenuity Pathway analysis. Consistent with this finding, these cell lines had markedly different proliferation rates, with *BRCA1*-deficient cells proliferating considerably faster than their isogenic *BRCA1*-proficient counterparts (Fig. 4). This suggests that the increased activity of the NF-κB and/or MAPK pathways in HCC1937 cells might play an important role in cell survival and proliferation in context of *BRCA1*.

TRAF2 is regulated by miR-146, miR-99 and miR-205

The computational analyses revealed *TRAF2* gene as a common target for five out of nine differentially expressed miRNAs, miR-146a/b, miR-99a/b and miR-205, with several predicted binding sites in its 3′UTR (Fig. 5a). In addition, we observed that *TRAF2* had higher endogenous expression, at both protein (Fig. 5d) and mRNA level (Fig. 6), in HCC1937, which expressed lower levels of miR-146a/b, miR-99a/b and miR-205. To validate *TRAF2* as a *bona fide* target of these miRNAs, we performed luciferase reporter assays to check binding of these miRNAs to the 3′UTR of *TRAF2* gene. Cells transfected with miR-146 showed 50% reduction in normalized luciferase signal compared to scramble control, while miR-99 and miR-205 transfected cells exhibited approximately 30% decrease in luciferase activity (Fig. 5b). Combination of all three miRNAs did not affect any further the expression of *TRAF2*.

To study the effect of miRNA on the *TRAF2* mRNA and protein level changes, we transfected HCC1937 with pre-miRNA oligonucleotides for miR-146a, miR-99b and miR-205. Although mRNA levels of *TRAF2* did not show a significant reduction after miR expression (Fig. 5c), western blot analyses revealed reduction in *TRAF2* protein levels in cells transfected with pre-miR-146a, pre-miR-205 and pre-miR-99b, compared to scramble control and mock transfection (Fig. 5d). Collectively, these luciferase and western blot results showed that miR-146a, miR-99b and miR-205 act as negative regulators of *TRAF2* expression.

NF-κB pathway activity is modulated by miR-146, miR-99 and miR-205

In order to determine whether miR-146a, miR-99b and miR-205 could have a direct effect on the NF-κB, MAPK/ERK and MAPK/JNK pathway activation, we re-expressed these miRNAs individually in HCC1937 cells and assayed for pathway activation using the pathway reporter assay. Our results demonstrated that all three miRNAs significantly reduced NF-κB pathway activity compared to negative control (Fig. 7). Similarly, MAPK/ERK pathway exhibited significant reduction in activation only upon transfection with miR-146a. On the other hand, MAPK/JNK pathway had similar level of activation as negative control, indicating that expression of just one miRNA was not sufficient to modulate activity of this pathway.

Down-regulation of miR-99a, miR146b and miR-205 in primary *BRCA1*-mutated breast tumors

To further explore the significance of miRNAs found to be associated with *BRCA1* re-expression in HCC1937, their

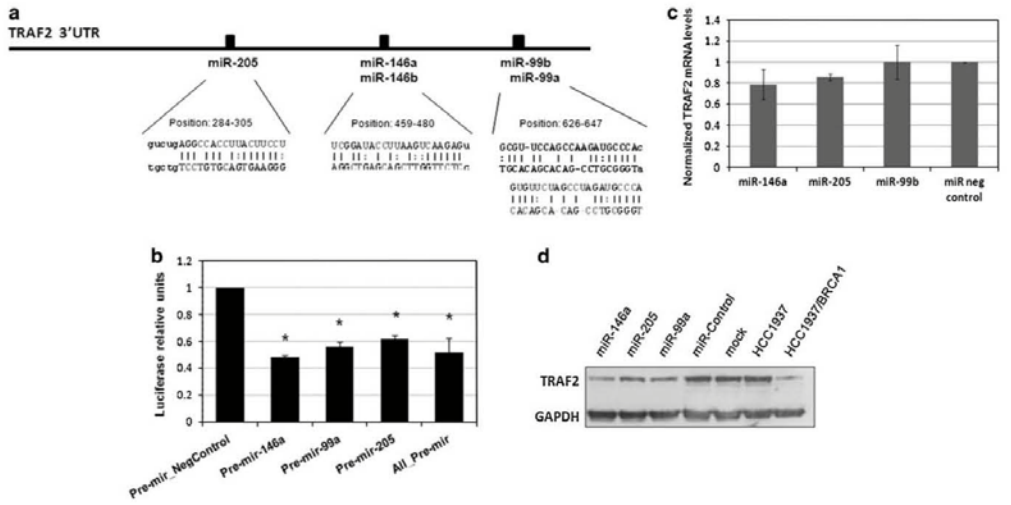

Fig. 5 Negative regulation of TRAF2 expression by miRNAs. **a** miRNA binding sites in TRAF2 3′UTR. Schematic representation of miRNA binding sites within the TRAF2 3′UTR region. Complementary binding sites for miR-146a, miR-146b, miR-99a, miR-99b and miR-205 are based on miRanda target prediction software. **b** Luciferase activity of a reporter construct carrying the TRAF2 3′-UTR downstream of the luciferase gene. The construct was co-transfected with each of the indicated miRNA precursors and pool combination of all 3 pre-miRNAs into HCC1937 cells. The pool contains an equimolar mixture of all indicated miRNAs in which the sum of all these miRNAs equals 50 nM as in the scramble vectors or the previous assays. All data are normalized versus the luciferase levels generated by scramble sequences. (*$P < 0.05$). **c** TRAF2 mRNA levels after miRNA expression. Reduction in the mRNA levels after miRNA expression comparing to the negative control showed no significant differences. **d** TRAF2 protein levels after miRNA expression. The effect of miR-146a, miR-99a and miR-205 on TRAF2 protein levels was assessed by western blot. HCC1937 cells were transfected with 50 nM miR-146a, miR-99a, miR-205 or scramble control, or mock-transfected. TRAF2 levels in non-transfected HCC1937 and HCC1397/BRCA1^{wt} are also represented. GAPDH was used as a loading control

level of expression was analyzed in a group of 13 *BRCA1* primary tumors comparing to expression in normal breast tissue. Significant down-regulation of three of these miRNAs, miR-99a, miR146b and miR-205 was confirmed in *BRCA1* tumors (Fig. 8), indicating that at least part of the *BRCA1*-associated miRNAs could be important also for *BRCA1*-mutated tumor development.

Discussion

In this study, we explored the effect of *BRCA1* on miRNA and gene expression profiles. After integration analysis of miRNA and mRNA signatures associated with *BRCA1* expression in HCC1937, significant interactions between three differentially up-regulated microRNAs (miR-146a/b and miR-99b) and down-regulated targets and signaling pathways were found. Bioinformatic predictions guided us to demonstrate that induced miRNAs after *BRCA1* re-expression, miR-146a, miR-99b and miR-205, regulate *TRAF2* and can modulate NF-κB activation. Thus, miRNA and mRNA data integration, in combination with pathway analysis, is a suitable approach for identifying novel miRNA target genes and pathways.

Reconstitution of *BRCA1* expression in *BRCA1*-null cells had profound impact on gene expression, with a large number of genes being regulated. These findings are in line with previously published results showing that depletion of *BRCA1* induces large changes in transcriptional profile [25, 30, 31]. Still, shift in gene expression could be also due to indirect effects of *BRCA1*, including potential gene expression regulation by miRNAs. Although *BRCA1* reconstitution modulated approximately one-third of genes, only 1% of analyzed miRNAs exhibited significant changes in expression.

Among miRNAs that were found to be up-regulated upon *BRCA1* reconstitution were miR-146a and miR-146b, which were previously reported to regulate the expression of *BRCA1* [32]. In addition, in silico predictions show a conserved binding site within *BRCA1* 3′UTR for miR-205, which was also significantly up-regulated upon *BRCA1* reconstitution. The mechanisms by which *BRCA1* may

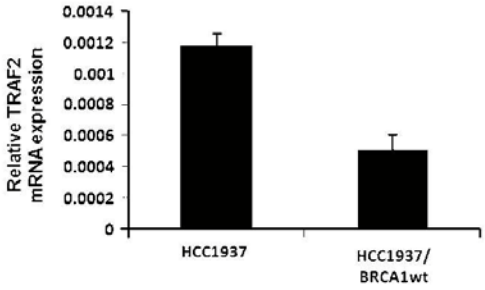

Fig. 6 mRNA levels of TRAF2 in HCC1937 and HCC1937/BRCA1wt cells. Endogenous TRAF2 mRNA levels are higher in BRCA1-null HCC1937 than HCC1937/BRCA1^{wt}. Bars represent mean expression values, and error bars represent standard deviation from three different experiments

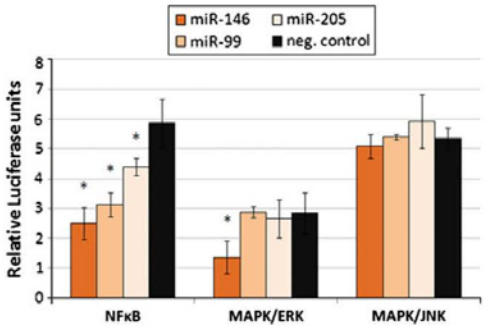

Fig. 7 miRNA expression effects on pathway activation. Effect of miR-146a/b, miR-199a/b and miR-205 on NFκB, MAPK/ERK and MAPK/JNK pathway activation was assessed by measuring the activities of downstream transcription factors in the BRCA1-deficient HCC1937 cell line transfected with 200 pm of each of these miRNAs. ($*P < 0.05$)

induce the expression of miRNAs could be either through direct or indirect effects and must be explored in further analysis.

One of the challenges in interpretation of miRNA profiling data is the identification of miRNA target genes due to large percentage of false-positive and false-negative results provided by available target prediction algorithms [33, 34]. In an effort to identify statistically significant miRNA target genes relevant to our dataset, we performed integration analysis based on non-random association of miRNAs with their negatively correlated predicted target genes. This approach revealed differentially expressed miRNAs (miR-99b, miR-146a and miR-146b) significantly associated with down-regulated mRNA targets. Gene set enrichment analysis allowed us to obtain an overview of key pathways likely to be modulated by BRCA1, among which were NF-κB, JNK and p38/MAPK pathways. Increased activation of MAPK/JNK in the BRCA1-null cell line was validated by in vitro pathway reporter assay, confirming at least part of the bioinformatic predictions.

The MAPK pathways are major signal transduction pathways that have been implicated in mammary epithelial cells and breast disease [35, 36]. Increased activation of MAPK/JNK and MAPK/ERK in *BRCA1*-deficient cells is consistent with higher proliferation rates exhibited by these cells. NF-κB is a transcription factor that promotes the expression of genes involved in inflammatory and anti-apoptotic response [37–40]. It has been demonstrated that NF-κB plays a critical role in development and progression of breast cancer [41–43]. Furthermore, it has been proposed that BRCA1 acts as a co-activator of NF-κB [44]. Interestingly, it has been previously reported that miR-146a/b acts as a negative regulator of constitutive NF-κB activity in breast cancer setting [45]. In addition, NF-κB-dependent induction of miR-146a/b has been established that, along with miR-146 negative regulation of IRAK1 and TRAF6, constitutes a negative-feedback loop that controls the activity of NF-κB pathway [46]. Here, we identify a novel target of miR-146a, *TRAF2*, through which it may exert

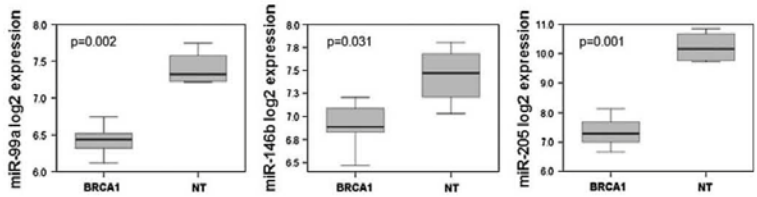

Fig. 8 Differential miRNAs expression between BRCA1 breast tumors and normal breast tissue. Box plots representing level of expression of a set of BRCA1-mutated tumors comparing to normal breast tissue (NT) for three significant miRNAs. MiR-99a, miR-146b and miR-205 showed significant down-regulation in tumors

it effects on NF-κB pathway regulation. This finding emphasizes the complex nature of feedback mechanism of regulation between miR-146a and NFκB through multiple facets, including both direct suppression of NFκB transcription factor expression and that of its mediators of activation, TRAF6, IRAK1 and newly identified TRAF2.

In support of the aforementioned findings, we have demonstrated that miR-146a/b along with newly identified miR-99b and miR-205 can modulate NF-κB pathway activation, while MAPK/ERK can be modulated only by miR-146a. On the other hand, individually none of these miRNAs were sufficient to modulate MAPK/JNK pathway whose activity was increased in BRCA1-null cells. This result could be explained by diverse and/or contrasting functions of many target genes for each individual miRNA, emphasizing the notion that different combinations of deregulated miRNAs could have very different biological outputs.

Consistent with the bioinformatic predictions, we have shown that TRAF2 had higher expression in *BRCA1*-null cells. Moreover, overexpression of genes related to immune response, including *TRAF2* gene, is one of the intrinsic characteristics of in ER-negative *BRCA1*-mutated tumors [47]. Numerous studies have implicated *TRAF2* as a critical mediator of NF-κB [48–53], JNK and p38 activation [51, 54, 55], and it has been reported that overexpressed native *TRAF2* gene can activate JNK, p38 and NF-κB in the absence of extracellular stimuli [52, 54, 56, 57]. Up-regulation of TRAF2 expression in BRCA1-deficient cells due to release of negative regulation by miR-146a/b, miR-99 and miR-205 could contribute to NF-κB and MAPK pathway activation. Significantly, further validation of microarray results, showing the up-regulation of miRNAs when *BRCA1* was introduced in HCC1937 BRCA1-null cells, came from our finding of at least three of these miRNAs, miR-99a, miR-146b and miR-205, being down-regulated in a set of BRCA1-mutated breast tumors.

In summary, we have established the global miRNA and mRNA expression pattern after BRCA1 reconstitution in HCC1937 cells and integrated the data to model differentially expressed miRNA and genes into functional networks. Our data suggest a role for *BRCA1* in modulating a number of miRNAs and indirectly hundreds of genes, in turn promoting the repression of NF-κB and MAPK signaling pathways. We have identified *TRAF2* as a novel target gene for miR-146, miR-99 and miR-205 and shown that these miRNAs are sufficient to modulate NF-κB pathway activity in breast cancer cells. Finally, our data underscore the importance of an integrated approach to study of miRNA and gene expression, for identification of altered signaling pathways and promising candidate genes. Down-regulation of these miRNAs also in primary tumors suggests their role in tumorigenesis and as potential targets for future treatment development.

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Conflict of interest The authors declare that they have no conflict of interest.

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Deregulated miRNAs in Hereditary Breast Cancer Revealed a Role for miR-30c in Regulating KRAS Oncogene

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Abstract

Aberrant miRNA expression has been previously established in breast cancer and has clinical relevance. However, no studies so far have defined miRNAs deregulated in hereditary breast tumors. In this study we investigated the role of miRNAs in hereditary breast tumors comparing with normal breast tissue. Global miRNA expression profiling using Exiqon microarrays was performed on 22 hereditary breast tumors and 15 non-tumoral breast tissues. We identified 19 miRNAs differentially expressed, most of them down-regulated in tumors. An important proportion of deregulated miRNAs in hereditary tumors were previously identified commonly deregulated in sporadic breast tumors. Under-expression of these miRNAs was validated by qRT-PCR in additional 18 sporadic breast tumors and their normal breast tissue counterparts. Pathway enrichment analysis revealed that deregulated miRNAs collectively targeted a number of genes belonging to signaling pathways such as MAPK, ErbB, mTOR, and those regulating cell motility or adhesion. *In silico* prediction detected KRAS oncogene as target of several deregulated miRNAs. In particular, we experimentally validated KRAS as a miR-30c target. Luciferase assays confirmed that miR-30c binds the 3'UTR of KRAS transcripts and expression of pre-miR-30c down-regulated KRAS mRNA and protein. Furthermore, miR-30c overexpression inhibited proliferation of breast cancer cells. Our results identify miRNAs associated to hereditary breast cancer, as well as miRNAs commonly miss-expressed in hereditary and sporadic tumors, suggesting common underlying mechanisms of tumor progression. In addition, we provide evidence that KRAS is a target of miR-30c, and that this miRNA suppresses breast cancer cell growth potentially through inhibition of KRAS signaling.

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Introduction

Breast cancer is the most common malignancy among women in developed countries. The majority of breast cancers are sporadic, while familial breast cancer comprises 5–10% of all breast cancers. Germline mutations in the currently known high risk-breast cancer genes (such as BRCA1/2) are common in familial breast cancer, but they can explain, at best, 20–25% of the overall excess familial risk. [1]. Still, the large majority of breast cancer cases that arise in families with strong familial aggregation are not explained by mutations in any know breast cancer susceptibility gene, and are designated as BRCAX-type tumors [2].

In the past decade, gene expression profiling by microarray analysis has lead to great advances in classification of human breast tumors, and the identification of five reproducible molecular subtypes of breast cancer, that have distinct biological features, clinical outcomes, and responses to chemotherapy [3]. On the other hand, there have been only a handful of studies focused on familial breast cancer, due to difficulties in collecting the tumor material, demonstrating that BRCA1/2-mutated breast tumors could be distinguished from sporadic ones based on their gene expression signatures [4].

Recently, microRNA (miRNA) expression profiling calls a great attention to define various types of cancers [5,6]. miRNAs are an abundant class of small ~22 nt long single-stranded non-coding RNA molecules acting as negative regulators at post-transcriptional level by binding the 3′ untranslated regions (3′UTRs) of their mRNA-targets [7]. miRNAs are involved in crucial biological processes including development, differentiation, apoptosis and proliferation [6]. Notably, miRNA deregulation has been extensively implicated in cancer pathogenesis in various tumor types [8,9]. The observed effects of miRNA mis-expression on tumor initiation, maintenance or metastasis can be explained by the mRNA targets and pathways they regulate, which include known tumor suppressors and oncogenes [10]. Specifically, in breast cancer, various studies have identified mis-expressed miRNAs in

tumours vs. normal tissue, and shown that changes in their expression seem to define, similarly to what has been found by expression profiling of coding genes, different histological (lobular/ductal, ER+/ER−) [11,12] and molecular (luminal A, luminal B, basal-like, HER2+) subtypes described so far [13]. In addition, integration of miRNA and mRNA data of a set of breast cancer samples allowed the association of miRNAs to relevant cellular processes, such as proliferation, cell cycle, immune response or cell adhesion, as well as with molecular characteristics of tumors like TP53 mutations [14].

Still, very little is known about the role of miRNAs in familial breast cancer. The identification of target genes and pathways regulated by miRNAs would be critical to understand their function in tumor development. In this study we sought to establish miRNA expression profiles using microarray technology of familial breast cancer tumors and comparing with normal breast tissues. Interestingly, KRAS has been identified as a target oncogene for down-regulated miRNAs. Direct regulation of KRAS by miR-30c and growth inhibition by this miRNA was experimentally demonstrated. The identification of miRNAs deregulated in familial breast tumors could provide a better understanding of the biology of familial breast cancer and could indicate novel targets for therapy.

Results

miRNA Expression Profiling in Primary Familial Breast Tumors and Normal Breast Tissue

In order to establish the miRNA profile of hereditary breast tumors, we used LNA based microRNA microarrays. After initial preprocessing we had data from 1276 hsa-miRNAs (831 hsa-miR and 434 hsa-miRPlus). A filter procedure to eliminate genes with low expression variation across the experiments (SD<0.3) and with uniformly low expression, reduced the number of miRNAs to a total of 327 hsa-miRNAs (198 hsa-miR and 118 miRPlus). In an effort to detect significant differences in miRNA expression between normal breast tissue and hereditary breast tumors, we performed a differential expression analysis. We identified 19 miRNA significantly differentially expressed (FDR<0.05) between normal breast tissue and familial tumoral samples (Figure 1). Almost all differentially expressed miRNAs were found to be down-regulated in tumor tissues, with the exception of miR-21 and miR-300 that were up-regulated in breast tumors compared to normal breast tissue (Table 1). Down-regulation of selected miRNAs was validated by qRT-PCR in an independent set of tumors (Figure S1).

Expression of these 19 differentially expressed miRNAs in normal Human Mammary Epithelial Cells (HMEC) was similar to the expression in normal breast tissue although some differences also exist for specific miRNAs. HMEC cells represent normal proliferating cells and normal tissue represent preferentially non proliferating cells, therefore, down regulation of some miRNAs, such as miR-99a, miR-101 or miR-145 (Figure 1), might be related to both normal and tumoral proliferation. However, down-regulation of other miRNAs, miR-205, miR-125a/b, miR-100 or miR-30c, might have a role in more specific tumoral processes.

miRNAs Commonly Deregulated in Familial and Sporadic Breast Cancer

Interestingly, several miRNAs that we found to be deregulated in familial breast cancer were previously described to be deregulated in sporadic breast tumors. Thus, 11 of the 19 miRNAs (miR-10b, -100, -101, -125a, -125b, 130a, -143, -145, -21, -205, and -30c) were previously identified in two key studies as being miss-regulated in sporadic breast tumors in comparison to normal tissue [11,12], suggesting that these miRNAs may play a general role in breast carcinogenesis (Figure 2). Deregulation of some of these miRNAs in sporadic tumors was also confirmed by qRT-PCR analysis in an independent set of 18 sporadic breast tumors (Figure S1).

Pathway Enrichment Analysis

Given the fact that a single miRNA can target a large number of mRNA transcripts, miss-expression of a set of miRNAs could have significant effect on cellular function by affecting multiple signaling pathways. To assess the potential impact of deregulated miRNA in hereditary breast tumors on biological processes and pathways, we used Diana miRPath web-based computational tool for biological interpretation of miRNA profiling data using over-representation analysis of biological processes and signaling pathways that are targeted collectively by co-expressed miRNAs. KEGG pathway enrichment analysis has revealed that the set of differentially expressed miRNAs between normal tissue and hereditary breast tumors, targets multiple effectors of pathways involved in ubiquitilation, cell proliferation and migration. Full list of pathways that have significantly overrepresented genes ($p<0.05$) collectively targeted by the set of 19 differentially expressed miRNAs is shown in Table 2. Observed down regulation of miRNAs in tumors regulating expression of these genes may result in abnormally activated pathways leading to increased proliferation and/or migration abilities.

We focused on the MAPK signaling pathway since a large number of genes within this pathway were found to be commonly targeted by 8 out of 19 deregulated miRNAs in our study suggesting that these miRNAs might cooperate to affect gene expression and consequentially activation or repression of signaling pathways (Table 2). Interestingly, miR-30c has potential binding sites on 20 different target genes involved in MAPK pathway (Table S1). This miRNA potentially target important mediators of MAPK signaling, such as KRAS, RASA1, MAP3K1 and MAPK8. Furthermore, KRAS gene has been previously validated as a target of several miRNAs, including let-7, miR-143 and miR-96 [15,16,17]. Now, we investigated whether miR-30c could be regulating KRAS expression in hereditary breast tumors. Thus, we confirmed by pRT-PCR that miR-30c had decreased expression in both hereditary and sporadic breast tumors comparing to normal samples (Figure 3). In addition, the other KRAS regulating miRNAs, let-7 and miRNA-143 were previously found to be significantly down-regulated in breast tumors [15,16], while miR-96 regulated KRAS in pancreatic tumors [17]. Although mutations in KRAS are infrequent in breast tumors, activation of KRAS pathway in breast cancer have been frequently found. All these data suggests that deregulation of miRNAs would be a mechanism to explain KRAS overexpression in breast tumors. The role of miR-143 and miR-145 in regulating KRAS expression was already described, however it was never described miR-30c targeting KRAS.

miR-30c Regulates KRAS Expression

We explored the role of miR-30c in regulation of KRAS expression. A negative correlation between miR-30c expression and KRAS protein level was observed in two breast cancer cell lines (Figure 4). In addition, several bioinformatic target prediction algorithms (DIANA microT4.0, TargetScan, PITA, PicTar, miRANDA) indicated existence of a broadly conserved putative binding site for miR-30c in the 3′UTR of the KRAS gene (Figure 5A). To test the hypothesis that KRAS is a bona fide target of miR-30c, we constructed a reporter plasmid harboring 300 pb

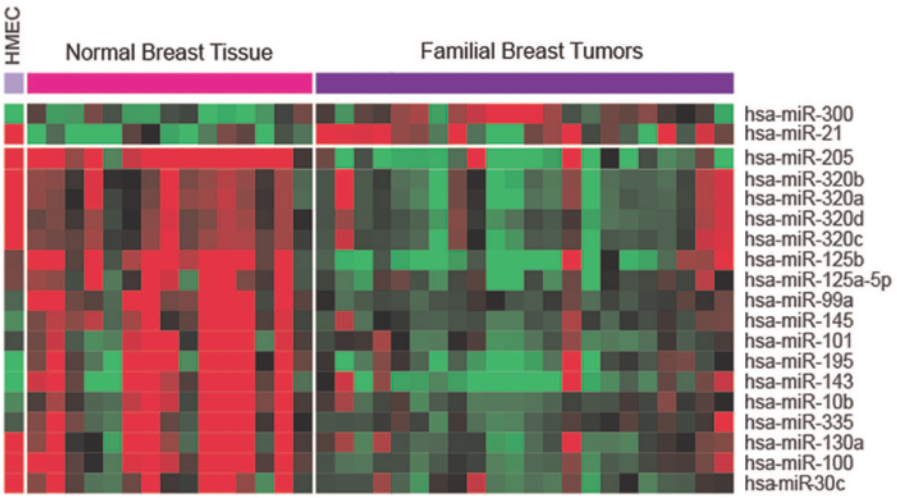

Figure 1. Differentially expressed miRNAs between normal breast tissue and hereditary breast tumors. Heat map of the expression of the 19 miRNAs differentially expressed between normal samples and tumors, overexpression in red, lower expression in green. Expression of these miRNAs in Human Mammary Epithelial Cells (HMEC) cells is also represented.
doi:10.1371/journal.pone.0038847.g001

of the wild-type 3′UTR region of KRAS flanking miR-30c binding site downstream of the luciferase coding region. MDA-MB-436 cells were co-transfected with luciferase reporter and pre-miR-30c or scramble control. As a result, pre-miR-30c transfected cells showed a marked reduction (52%) of luciferase activity compared to scramble control, confirming the interaction between

Table 1. miRNAs differentially expressed between normal breast and familial tumor tissue.

miRNA	Chromosomal location	Median Normal Breast	Median Tumor Tissue	Fold change	Unadjusted p value	FDR[1]
hsa-miR-205	1q32.2	9.9	7.4	5.9 ↓	3.00E−07	7.82E−05
hsa-miR-125b	11q24.1/21q11.2	11.4	8.9	5.8 ↓	6.00E−07	7.82E−05
hsa-miR-99a	21q11.2	7.4	6.3	2.2 ↓	1.30E−06	0.0001
hsa-miR-100	11q24.1	7.6	6.4	2.2 ↓	2.00E−06	0.0001
hsa-miR-145	5q32−33	7.3	6.4	1.8 ↓	4.95E−05	0.0024
hsa-miR-195	17p13	9.1	7.4	3.4 ↓	0.000152	0.0062
hsa-miR-10b	2q31	7.6	6.8	1.7 ↓	0.00024	0.0084
hsa-miR-320c	18q11.2	7.6	6.9	1.6 ↓	0.000412	0.0127
hsa-miR-320d	13q14.11/Xq27.1	7.4	6.7	1.6 ↓	0.000512	0.0140
hsa-miR-101	1p31.3	7.4	6.4	2.0 ↓	0.000787	0.0185
hsa-miR-130a	11q12	7.5	6.8	1.6 ↓	0.000887	0.0185
hsa-miR-320b	1p13.1/1q42.11	8.1	7.3	1.8 ↓	0.000938	0.0185
hsa-miR-125a-5p	19q13.4	8.8	8.1	1.6 ↓	0.000976	0.0185
hsa-miR-335	7q32.2	7.1	6.5	1.4 ↓	0.001195	0.0210
hsa-miR-320a	8p21.3	8.1	7.3	1.8 ↓	0.001669	0.0257
hsa-miR-143	5q32−33	9.7	8.2	2.8 ↓	0.002078	0.0301
hsa-miR-21	17q23.1	9.1	10.1	2.0 ↑	0.003822	0.0495
hsa-miR-30c	6q13	8.4	7.8	1.5 ↓	0.004283	0.0504
hsa-miR-300	14q32.31	7.1	8.0	2.0 ↑	0.004306	0.0504

[1]FDR: False discovery rate adjusted p value.
doi:10.1371/journal.pone.0038847.t001

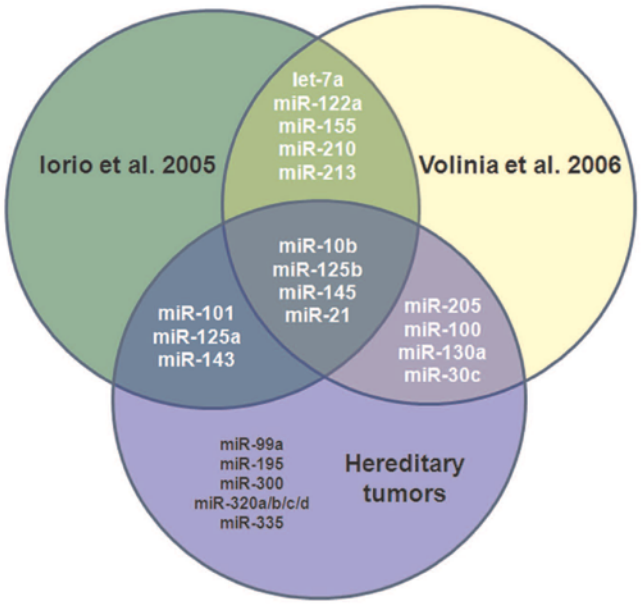

Figure 2. Shared differentially expressed miRNAs in sporadic breast tumors and hereditary tumors. Venn diagrams representing commonly deregulated miRNAs in two different studies carried out in sporadic breast cancer samples [11,12] and in the present study on hereditary breast tumors. Regardless of the genetic background or histopathological features of the tumors, there are miRNAs consistently altered in breast tumor samples.
doi:10.1371/journal.pone.0038847.g002

Table 2. Significantly enriched signaling pathways associated to the differentially expressed. miRNAs.

KEGG Pathway	N° of miRNA Target Genes	-LN(p-value)	Gene Names
Ubiquitin mediated proteolysis	20	17.18	UBE2D1, SOCS1, UBE2D2, UBE3C, UBE1, MAP3K1, BIRC6, UBE2J1, UBE2I, SMURF1, UBE2W, CBL, BTRC, WWP1, CUL2, SOCS3, CBLB, NEDD4L, NEDD4, ITCH
Axon guidance	17	11.88	SRGAP3, PLXNA2, GNAI2, DPYSL2, ITGB1, UNC5C, EFNA3, SEMA4D, EPHB2, SEMA6D, KRAS, CFL2, NRP1, PPP3CA, RASA1, SEMA3A, NFAT5
Insulin signaling pathway	16	7.96	PPARGC1A, SOCS1, MAPK8, TSC1, PDPK1, KRAS, CBL, FRAP1, CRKL, SORBS1, SOS1, FOXO1, SOCS3, CBLB, PIK3CD, AKT3
O-Glycan biosynthesis	6	7.23	GALNT2, GALNT7, GALNT1, GALNT3, GCNT1, B4GALT5
mTOR signaling pathway	7	5.31	TSC1, PDPK1, FRAP1, ENSG00000164327, RPS6KA3, PIK3CD, AKT3
ErbB signaling pathway	10	4.74	MAPK8, NRG3, KRAS, CBL, FRAP1, CRKL, SOS1, CBLB, PIK3CD, AKT3
Glycan structures - biosynthesis 1	12	4.35	GALNT2, GALNT7, GALNT1, GALNT3, CHST2, STT3B, CHST1, GCNT1, EXTL2, B4GALT5, MAN1A2, XYLT1
MAPK signaling pathway	20	3.84	MAP4K4, MAPK8, MAP3K1, KRAS, MEF2C, BDNF, CRKL, TAOK1, STK4, SOS1, FGFR1, RPS6KA5, FGF2, RAP1B, PPP3CA, NF1, RASA1, MAP3K12, RPS6KA3, AKT3
Regulation of actin cytoskeleton	17	3.61	ITGB1, WASL, ARHGEF6, KRAS, PIP4K2B, CRKL, CFL2, PIP4K2A, ITGA6, SOS1, FGFR1, FGF2, ITGB3, GNA13, ACTC1, PIK3CD, PFN2
Adherents junction	8	3.55	IGF1R, SNAI1, WASL, SMAD2, SORBS1, FGFR1, SSX2IP, PVRL1
Focal adhesion	16	3.54	BCL2, MAPK8, ITGB1, IGF1R, PDPK1, CRKL, ITGA6, SOS1, PTEN,PTENP1, RAP1B, ITGB3, ARHGAP5, CCND2, PIK3CD, AKT3, CCND1
T cell receptor signaling pathway	9	3.2	KRAS, CBL, SOS1, PPP3CA, CBLB, PDCD1, NFAT5, PIK3CD, AKT3

doi:10.1371/journal.pone.0038847.t002

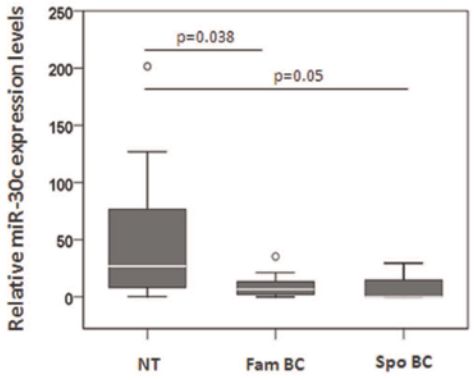

Figure 3. Validation of miR-30c expression by qRT-PCR in hereditary and sporadic tumors. Expression levels of miR-30c in an independent set of 12 familial (FamBC) and 8 sporadic tumors (SpoBC) comparing to normal breast tissue expression. Differences were estimated by t-test and *p* values are shown for each comparison.
doi:10.1371/journal.pone.0038847.g003

miR-30c and KRAS 3′UTR binding site (Figure 5B). Next, we checked whether miR-30c could affect KRAS mRNA stability by performing qRT-PCR analysis in MDA-MB-436 cells transiently transfected with either pre-miR-30c or scramble control. Indeed, we observed a sharp decrease in KRAS mRNA levels upon transfections with pre-miR-30c (Figure 5C).

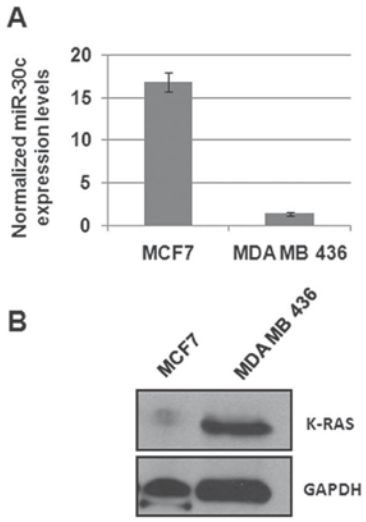

Figure 4. Correlation of expression of miR-30c and KRAS in two breast cancer cell lines. Inverse correlation between the expression level of miR-30 determine by qRT-PCR (A) and detection of KRAS protein in MCF7 and MDA-MB-436 cells (B).
doi:10.1371/journal.pone.0038847.g004

In addition, pre-miR-30c or pre-miR- control were transfected into MDA-MB-436 cells, and we confirmed a reduction of about 44% of KRAS protein level in MDA-MB-436 cells over-expressing miR-30c, in comparison to control (Figure 5D).

miR-30c Affects Proliferation of Breast Cancer Cells

To gain more insight into the biological effects of miR-30c on breast tumorigenesis and given that KRAS plays a role in regulation of cell proliferation, MDA-MB-436 cells, which previously showed elevated levels of KRAS protein, were transfected with pre-miR-30c or scramble and analyzed for cell growth. As shown in Figure 5E, ectopic expression of miR-30c resulted in reduced proliferation in comparison to scramble control transfected cells. Therefore, modulation of KRAS protein level by miR-30c may explain at least in part, why down-regulation of miR-30c can promote proliferation and contribute to tumorigenesis.

Discussion

miRNA profiling studies in various tumor types have demonstrated a widespread miRNA deregulation, providing for new insights in tumor biology, improved classification and opening new avenues for development of novel targeted therapies in cancer. In this study we performed miRNA expression profiling in familial breast tumors and normal breast tissue in order to uncover deregulated miRNAs. The normal breast tissue series was obtained from healthy individuals, both from BRCA1/2- mutation carriers belonging to high risk families, and from mutation-free individuals undergoing breast reduction surgery. This has enabled us to pinpoint miRNAs important for tumorigenesis, by removing the bias induced through effects of BRCA1/2 mutation on miRNA expression. We have defined down-regulation for 17 miRNAs and up-regulation of miR-21 and miR-300 in familial breast tumors when comparing to normal breast tissue.

A high proportion of deregulated miRNAs in hereditary tumors were commonly found in previous studies with sporadic breast tumors, suggesting that there are miRNAs that likely regulate important oncogenes involved in both familial and sporadic tumors, irrespective of their genetic background. It was expected that comparing tumor samples with normal tissue, which is characterized by quiescent cells, would uncover miRNAs that may be mainly involved in proliferation. Expression analysis of the 19 differentially expressed miRNAs in HMEC cells, which represent normal proliferating cells, have allowed finding some miRNAs overexpressed in normal tissues that were not overexpressed in normal HMEC cells, such as miR-99a, miR-101 or miR-145, which could have a role in the control of proliferation processes in general. Other miRNAs expressed by both HMEC and normal breast and down-regulated in tumors tissue may be involved in other signaling pathways related to tumoral behavior.

Moreover, the pathway enrichment analysis suggested that co-expressed miRNAs seem to collectively target a broad range of signaling pathways related to proliferation and cell migration/motility. Altered cell signaling has long been recognized as a mechanism employed by cells in the development and progression of cancer [18]. Importantly, 20 genes within MAPK signaling pathway were significantly associated with our set of deregulated miRNAs, suggesting that the inhibition of these miRNAs would result in a concomitant activation of MAPK signaling.

In breast cancer, MAPKs play a key role in transducing growth signals from the extracellular environment [19,20]. The activation of the KRAS/MAPK pathway generates a plethora of responses in breast cancer tumors and cell lines, affecting cell growth,

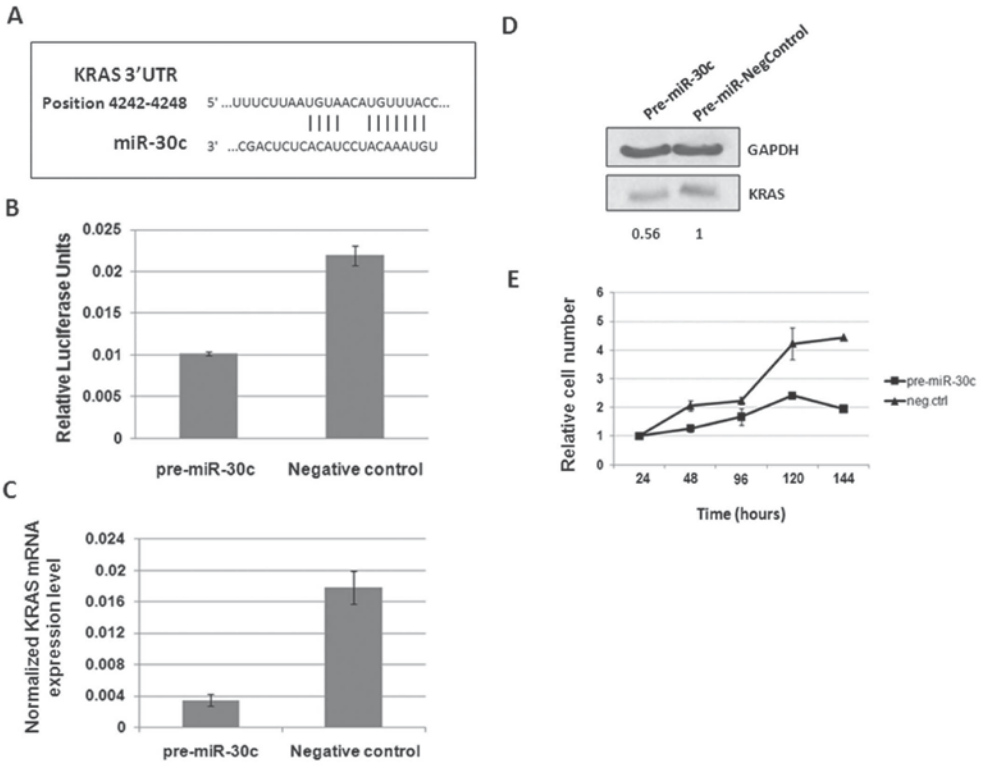

Figure 5. miR-30c effects on KRAS expression and cell proliferation. (A) Schematic representation of miR-30c binding site within the KRAS 3'UTR region. (B) Luciferase activity of a reporter construct carrying the KRAS 3'UTR downstream of the luciferase gene. The construct was co-transfected with pre-miR-30c or scramble control in MDA-MB-436 cells. (C) KRAS expression at transcription level. Significant reduced level of KRAS mRNA expression was detected by qRT-PCR after pre-miR-30c transfection, comparing with scramble control. (D) Regulation of KRAS protein level by miR-30c. MDA-MB-436 cells were transfected with pre-miR-30c or pre-miR-scramble oligonucleotides. After 48 hours KRAS protein was evaluated by western blot. GAPDH was used as loading control. The signal in each line was quantified and the ratio of KRAS to GAPDH was determined. (E) Effect of miR-30c expression on proliferation of MDA-MB-436 cells. MTT cell viability assay was performed at 48, 72, 96, 120 or 144 hours after transfection of MDA-MB-436 cells with pre-miR-30c or pre-miR-scramble oligonucleotides.
doi:10.1371/journal.pone.0038847.g005

proliferation, differentiation and transformation [21]. In breast cancer development, up-regulation of the KRAS/MAPK signaling can occur through multiple facets, and it has been shown to be increased in many breast cancer samples either by over-expression of growth-factor-receptor tyrosine kinases primarily HER2/ErbB-2, EGFR, and IGFR or by activating mutations [22,23,24,25]. Although KRAS is frequently mutated in human cancers including pancreatic, colorectal and lung cancers, KRAS mutations are extremely rare in breast cancer [26,27]. However wild-type KRAS is significantly activated in breast cancers that over-express EGFR and ErbB2 [28]. Additionally many investigators have reported over-expression of the KRAS-encoded p21 proteins in breast malignancies in comparison to normal breast tissue [29,30] although the role of this over-expression in breast carcinogenesis has not been determined.

Interestingly, KRAS was found to be a target of multiple miRNAs found to be down-regulated in breast tumors. The let-7 family of miRNAs has been shown to regulate multiple oncogenes, including KRAS and c-MYC [16], and miR-143/145 are involved in feed-forward mechanism that potentiates Ras signaling through down-regulation of KRAS and Ras-responsive element-binding protein (RREB1), which represses the miR-143/145 promoter [31]. Here we have identified a novel broadly conserved miRNA, miR-30c, as a direct negative regulator of KRAS expression. Interestingly, miR-30 and let-7 were reported to be markedly reduced in breast tumor-initiating cells and contribute to their self-renewal capacity and undifferentiated state, and ectopic expression of these miRNAs in breast tumor-initiating cell xenografts decreases their tumorigenic and metastatic potential [32]. Furthermore, it has been shown recently that higher expression of miR-30c was significantly associated to benefit of tamoxifen treatment and with longer progression-free survival [33]. Altogether, decreased expression of these miRNAs may release the negative regulation of KRAS. Interestingly, our results showed

that at least three KRAS regulating miRNAs (miR-30c, miR-143, and miR-145) had significantly reduced co-expression in tumors and then these miRNAs may act together in the regulation of KRAS oncogene.

Signal transduction pathways integrate signals from extracellular stimuli including mitogens, growth factors, hormones and environmental stresses- signals required for tumorigenesis. miRNA deregulation results in the complex modulation of multiple targets belonging to multiple pathways. Commonly deregulated miRNAs in both familial and sporadic breast cancer suggest that commonly altered pathways could be important for tumor progression. Here, we have demonstrated that KRAS inhibition through direct regulation by miR-30c leads to reduced proliferation in breast cancer cells. Similarly, other studies have identified KRAS as a target of several miRNAs down-regulated in tumors (let-7, miR-96 and miR-143), that also have an effect on cancer cell proliferation and tumor invasiveness [15,16,17]. Therefore, co-ordinated down-regulation of miRNAs found in breast tumors would be not only affecting KRAS oncogene expression but also may be targeting other genes of the KRAS/MAPK signaling pathway to cooperatively activate tumorigenic downstream signals.

In general a strong similarity between deregulated miRNAs was found in hereditary and sporadic breast cancer when compared to normal breast tissue. However, in order to get miRNAs associated with BRCA1 and BRCA2 mutated tumors, a higher number of BRCA1/2 mutated tumors would be needed. In this regard, one study found very similar miRNA expression profiles in high grade serous ovarian carcinomas with or without BRCA1/2 mutations [34]. More studies are guaranteed to determine the role of miRNA more related to familial breast tumors and those specifically associated to the BRCA1 and BRCA2 mutated tumors.

In summary, our data defined a deregulated set of miRNAs in hereditary breast tumors, many of them commonly deregulated in sporadic breast cancer. These miRNAs mostly showed significant reduced expression in tumors comparing to normal breast tissue. One of these miRNAs, miR-30c, potentially contributes to breast malignancy formation through release of KRAS suppression suggesting that this miRNA, and likely other miRNAs also targeting KRAS/MAPK signaling, may function as tumor suppressors in breast cancer.

Materials and Methods

Ethics Statement

Informed written consent was obtained from all patients involved in this study to perform genetic studies and to use exceeding material for research, and the research project has the approval of the ethics committee of the Spanish National Cancer Research Centre (CNIO), named *Comité de ética de la investigación y de bienestar animal del Instituto de Salud Carlos III.*

Samples

Tumor tissue samples were obtained from patients undergoing surgery for breast cancer from different Hospitals in Spain. Potential differences regarding ethnicity was not affecting in this case since all tumors were obtained from Spaniards patients. All patients belong to high-risk families with at least three members affected with breast and/or ovarian cancer and at least one of whom was younger than 50 years when diagnosed. For microarray analysis we included whole tissue sections from 22 frozen hereditary breast tumors consisting of 3 BRCA1-mutated, 5 BRCA2 and 14 non-BRCA1/2 (BRCAX) samples, and 14 normal breast tissues including 3 from BRCA1-mutation carriers, 5 from BRCA2-mutation carriers. Histopatological features of the tumors are shown in Table S2, 1 normal breast tissue from contra-lateral breast of patient with BRCAX tumor, and 5 normal breast tissues. Normal breast tissues were obtained after breast reduction surgery from healthy individuals with no family history of breast cancer, and normal breast tissue from BRCA1/2-mutation carriers were obtained after prophylactic surgery. The tissue collection used for validation included 18 paired samples from fresh sporadic breast tumors and their adjacent normal breast tissue counterparts, RNA from 6 additional and 4 normal breast tissue samples was also used for validation. In addition, expression of miR-30c expression was analyzed by qRT-PCR using RNA from FFPE tumor (12 hereditary and 8 sporadic breast tumors) and 9 breast tissue samples.

Normal Human Mammary Epithelial Cells (HMEC) (Clonetics) were used to evaluate miRNA expression comparing with normal breast tissue. HMEC cells were grown in MEGM, Mammary Epithelial Growth Medium (Clonetics) supplemented with growth factors SingleQuots (Clonetics) in absence of FBS.

miRNA Microarray

Total RNA was extracted from primary tumors using Trizol (Invitrogen). RNA quantity and quality were assessed by NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. Microarray expression profiling was performed using miRCURY LNA^{TM} microRNA Array kit (Exiqon A/S, Denmark) according to manufacturer's instructions, in a one-color, pair-wise comparison experimental design. Briefly, 300 ng of total RNA was labeled with Hy3 fluorescent dye and subsequently hybridized over 16 h at 56°C onto a miRNA microarray chip (v.11.0– hsa, mmu & rno) containing 1940 capture probes, in 4 replicates, representing 831 human miRNAs annotated in miRBasev.11 database. A set of 10 synthetic Spike-in RNAs was added to total RNA sample prior to labeling and later used for quality control. Processed slides were scanned with Agilent Array scanner (Agilent Technologies), with the laser set to 635 nm, at Power 80 and PMT 70 setting, and a scan resolution of 10 μm. Fluorescence intensities on scanned images were quantified using Feature Extraction software (Agilent Technologies) using the modified Exiqon protocol. Average values of the replicate spots were background subtracted and log transformed and subjected to further analysis. Microarray dataset is publicly available at GEO database http://www.ncbi.nlm.nih.gov/geo/info/linking.html under GEO accession number GSE32922.

Array Data Processing and Statistical Analysis

Raw data were quantile normalized for inter-array variability. Data was preprocessed to eliminate miRNAs with uniformly low expression or with low expression variation (SD<0.3) across the experiments, retaining 466 miRNA genes (306 hsa-miR +160 hsa-miRPlus). Average linkage hierarchical clustering (Pearson correlation, uncentered metrics) from Gene Cluster and Treeview (http://rana.stanford.edu/software) algorithms were used to obtain clustering of the data sets. To determine if there were genes differentially expressed between tumor and normal breast tissue, differential expression analysis was performed using t-test implemented in the POMELO II tool, available in Asterias web server (http://asterias.bioinfo.cnio.es) [35]. The estimated significance level were obtained by permutation testing and p-values were corrected for multiple hypotheses testing using Benjamini & Hochberg False Discovery Rate (FDR) adjustment [36]. Those

miRNAs with FDR <0.05 were selected as significantly differentially expressed.

Quantitative RT-PCR Analysis

Quantitative RT-PCR analysis of miR-30c, miR-100, miR-125b and miR-320a was performed on independent series of familial tumors as well as sporadic breast tumor samples and compared to normal breast tissue samples using miRCURY LNA™ microRNA PCR System (Exiqon). Briefly, 10 ng of total RNA was reverse-transcribed with miRNA specific primers and Transcriptor Reverse Transcriptase, and the cDNA was used as a template for the qPCR reaction using miRNA specific LNA™ PCR primer and Universal PCR primer. Gene expression levels were quantified using the ABI Prism Sequence Detection System 7900HT (Applied Biosystems). All experiments were performed in triplicate and the mean of triplicates was used. Normalization was done with SNORA66 RNA and 5S rRNA. Relative expression was calculated using the comparative Ct method.

Pathway Enrichment Analysis

DIANA miRPath pathway enrichment analysis (http://diana.cslab.ece.ntua.gr/) was used to gain insight into global molecular networks and canonical pathways related to differentially expressed miRNAs between normal and tumor samples. DIANA miRPath is a web-based computational tool developed to identify molecular pathways potentially altered by the expression of single or multiple microRNAs. The software performs an enrichment analysis of multiple microRNA target genes comparing each set of microRNA targets to all known KEGG pathways. Those pathways showing p-value <0.05, were considered significantly enriched between classes under comparison.

Cell Culture, Constructs and Transfections

The MDA-MB-436 was kindly provided by Dr. K.S Massey-Brown from Department of Pharmacology and Toxicology, University of Arizona, Tucson, USA, and was characterized in previous studies by our group [37]. The MDA-MB-436 cell line was cultured in RPMI 1640 medium supplemented with 10% FBS and 100 units/ml of Penicillin G and streptomycin. Cells were split 24 h prior to all transfection assays at a confluence 40–70%. Pre-miRNA oligonucleotides (pre-miR-30c and scramble control) were purchased from Ambion (Austin, Texas, USA). For luciferase reporter assay transfection of oligonucleotides and plasmids was performed using Lipofectamine 2000 according to the protocols provided by the manufacturer (Invitrogen, Calsbad, CA). For proliferation, and KRAS western blot analysis, pre-miRNA oligonucleotides were transfected to a 50 nM final concentration using Oligofectamine (Invitrogen).

KRAS-3′UTR Luciferase Reporter Assay

3′UTR sequence of the KRAS was retrieved through NCBI nucleotide database. A 300 bp fragment of the 3′UTR region of KRAS gene containing miR-30c binding site, was amplified by PCR from human genomic DNA, and cloned into a modified pGL3-Control vector (Promega) at the SacII and EcoRI site, immediately downstream of the luciferase stop codon. Primer sequences used to amplify this region were RAS3UTR-F: 5′CAC*GAATTC* CACACCCCCACAGAGCTAAC3′ and RAS3UTR-R: 5′TTCCCGCGGTGTTTGATATGACCAACATTCCT 3′. Correct vector construction was verified by direct sequencing. Dual luciferase assay was carried out by co-transfecting MDA-MB-436 cells with 25 pmol of pre-miR-30c or scramble control, along with 500 ng of KRAS 3′UTR- firefly luciferase construct and 7.5 ng of *Renilla* luciferase vector, using Lipofectamine 2000 (Invitrogen) per well, according to the manufacturer's protocol in a 24-well plate format. Cells were grown for 48 h, after which luciferase activity was assayed with Dual-Luciferase Assay System (Promega). Experiments were performed in triplicate and *Renilla* luciferase activity was used for transfection variation normalization.

Detection of KRAS mRNA and Protein Levels

To detect gene expression levels of KRAS, one μg of total RNA was reverse transcribed using MMLV Reverse Transcriptase (Invitrogen) and random primers. The cDNAs were subjected to quantitative RT-PCR assay with the use of labeled probes (Roche Universal Probe library) and the TaqMan Universal PCR Mix in an ABI Prism Sequence Detection System 7900HT (Applied Biosystems) under manufacturer's recommendations. The PCR amplification was carried out with 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C, using the oligonucleotides shown in Table S3. ß-actin was used as internal control and allowed normalization of the samples. All experiments were analyzed in triplicate.

Western blot analysis was performed using standard procedures for whole-cell extracts from cell lines. Lysates were prepared using RIPA buffer (Sigma-Aldrich). Equal amounts of protein lysates (50–100 μg) were separated by SDS-PAGE on 4–12% pre-casted gels (Invitrogen), and electrotransferred to nitrocelulose membranes (Wathman) and probed with primary antibody. Antibodies used were KRAS (F234, Santa Cruz) and GAPDH (CNIO, Monoclonal Antibodies Unit). Secondary antibody staining was carried out with anti-mouse HRP IgG (Dakko) and HPR activity was detected with ECL Detection System (GA Healthcare).

Cell Proliferation Assay

Cell viability was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MDA-MB-436 cells were transfected in independent experiments with pre-miR-30c/pre-miR-Scramble oligonucleotides. Cells were incubated in 1 μg/μl MTT Formazan (Sigma-Aldrich) diluted in normal culture medium at 37°C for 5 h. Cell viability was determined at 24, 48, 72, 96, 120 or 144 hours after transfection.

Supporting Information

Figure S1 Validation of miRNA expression by qRT-PCR in hereditary and sporadic tumors. Expression levels of miR-125b, miR-100, miR-320a in normal breast tissue comparing with familial tumor samples (FamBC) and sporadic breast cancer (SpoBC). Differences were estimated by t-test and *p* values are shown for each case.
(DOC)

Table S1 Predicted genes within MAPK pathway targeted by deregulated miRNAs in hereditary breast cancer tumors.
(DOC)

Table S2 Histopathological data from hereditary breast tumors.
(DOC)

Table S3 Primers used for measuring KRAS expression by quantitative RT-PCR (used with Universal ProbeLibrary probe#62, Roche).
(DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: BMD JB MT. Performed the experiments: MT KY. Analyzed the data: MT KY CRA RA IMR AO JB BMD. Contributed reagents/materials/analysis tools: CRA RA IMR. Wrote the paper: MT KY JB BMD.

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